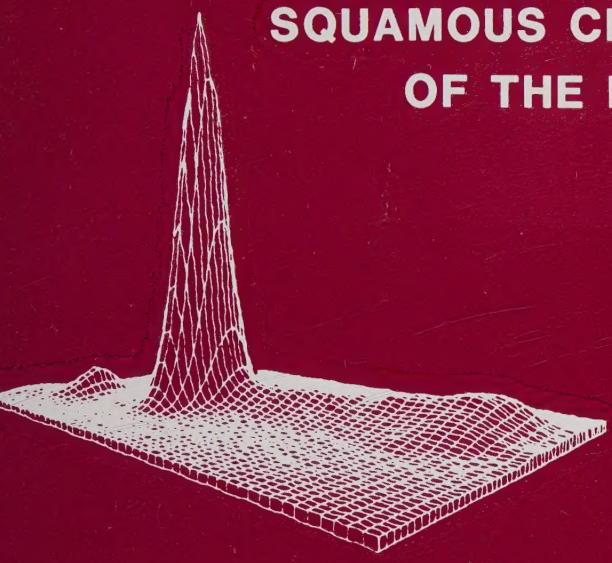
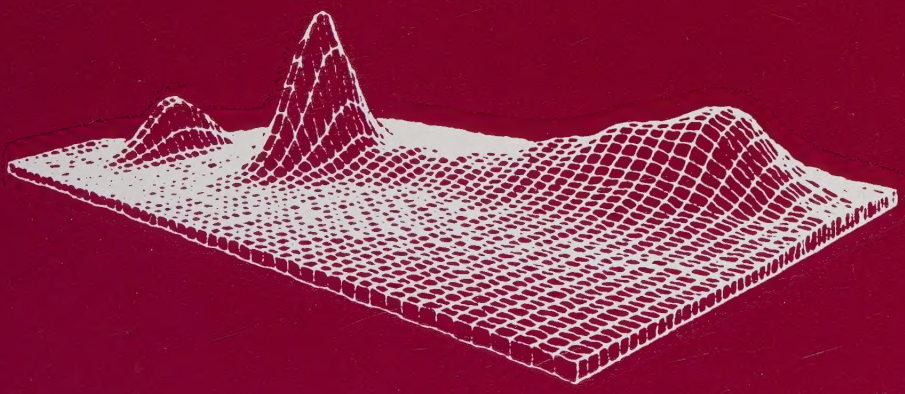
TUMOUR CELL KINETIC STUDIES IN HETEROTRANSPLANTED SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Johan Wennerberg





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Abstract

In the treatment of advanced squamous cell carcinoma (SCC) of the head and neck (H&N) combination chemotherapy is used. Analysis of chemotherapeutically induced changes in growth and cell cycle parameters under controlled conditions may provide a better basis for such therapy. Human SCC of the H&N transplanted to athymic, nude mice may provide a suitable model. In the present investigation this model has been evaluated with special reference to factors influencing take rate, cell kinetic changes induced by transplantation of tumour tissue from man to nude mice, and the occurrence of β_2-microglobulin (β_2m) in heterotransplanted SCC and SCC in man. Changes in growth and cell kinetic parameters induced by cytostatic drugs were also studied.

One third of the transplanted tumours grew in the first passage. Only 1/5 of attempted transplantations resulted in transplantable tumour lines. Take rate was not dependent on whether transplanted tumour tissues were obtained as "unsterile" diagnostic biopsies or "sterile" operative specimens. Advanced tumours (stage IV) were associated with a higher take rate than less advanced tumours.

During the first three to five passages of heterotransplants in nude mice there was an increase in 3HTdR incorporation into DNA and a decrease of tumour volume doubling time (DT) towards a constant value.

Flow cytometric (FCM) analysis of ploidy revealed that in tumours with one diploid and one aneuploid malignant cell population only the aneuploid populations could be recovered from the growing heterotransplants. β_2m could be detected in plasma of patients with SCC and tumour-bearing nude mice and was also detected immunohistochemically bound to cell membranes. In heterotransplanted tumours there was a correlation between DT and concentration of extractable β_2m.

The effect of cisplatin, mitomycin C and bleomycin on heterotransplanted tumours was investigated. All drugs exhibited a dose-response relationship, but the shape of the curves differed between the drugs. With FCM analysis of cell cycle phase distributions it was possible to detect significant but transient disturbances in the transition of the cells through the cell cycle.

The present investigation provides information on important differences between heterotransplanted SCC and SCC in man. Such knowledge is important for the design and correct interpretation of experiments on heterotransplanted tumours. In addition, by stepwise evaluation of single drugs in the nude mouse system, as demonstrated in the study, it is possible to gain knowledge of the course of induced cell cycle perturbations which can provide a basis for the design of combination chemotherapy.

Key words Cell kinetics, flow cytometry, DNA-synthesis, β_2-microglobulin, squamous cell carcinoma, head and neck cancer, athymic, nude mice, heterotransplantation, chemotherapy, cisplatin, mitomycin C, bleomycin.

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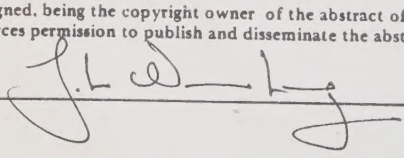
Signature 

Date 6 december 1983

TUMOUR CELL KINETIC STUDIES IN HETEROTRANSPLANTED SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Lund 1984

"It's impossible to make
things foolproof, because
fools are so ingenious."

Murphy's 8th law

To Gunilla, Henrik and Erik

The present thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I Wennerberg J, Tropé C & Biörklund A.
Heterotransplantation of human head and neck tumours into nude mice.
Acta Oto-Laryngologica 95:183-190, 1983.

II Wennerberg J.
Changes in growth pattern of human squamous cell carcinomas of the head and neck during serial passages in nude mice.
Accepted for publication in Int J Cancer.

III Wennerberg J, Alm P, Lögdberg L & Tropé C.
β_2-microglobulin in squamous cell carcinomas of the head and neck and in tumours heterotransplanted into nude athymic mice.
Accepted for publication in Acta Oto-Laryngologica

IV Wennerberg J, Alm P, Biörklund A, Killander D, Långström E & Tropé C.
Cell cycle perturbations in heterotransplanted squamous cell carcinoma of the head and neck after mitomycin C and cisplatin treatment.
Accepted for publication in Int J Cancer.

V Wennerberg J.
Bleomycin induced changes in growth and cell kinetics of a heterotransplanted squamous cell carcinoma of the head and neck.
Submitted for publication.

Abbreviations

β_2m	β_2-microglobulin
BLM	Bleomycin
CDDP	Cis-diamminedichloroplatinum II (cisplatin)
CPM	Counts per minute
DT	Tumour volume doubling time
FCM	Flow cytometry
GF	Growth fraction
3HTdR	Methyl-3H-thymidine (tritiated thymidine)
I.p.	Intra peritoneal
L-compartm.	Lethal - compartment
LD	Lethal dose
MMC	Mitomycin C
P-compartm.	Proliferative-compartment
Q-compartm.	Quiescent (non-proliferative)-compartment
RTS	Relative tumour size
S.c.	Subcutaneous
SCC	Squamous cell carcinoma
S.D.	Standard deviation
SEM	Standard error of mean
Tc	Cell cycle time

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Introduction

Head and neck carcinomas are uncommon compared to, for example, carcinoma in the breast and prostate. In Sweden 1979 a total of 34.255 cancers were registered in a population of 8.3 million. The incidence of head and neck carcinoma for the same year is given in Table I.

Table I. *Incidence of head and neck cancer*

Lip	194
Tongue	91
Salivary glands	81
Floor of mouth	27
Mouth, other parts	126
Mesopharynx	51
Nasopharynx	51
Hypopharynx	68
Nose and paranasal sinuses	45
Larynx	203
Thyroid gland	379

(Cancer Incidence in Sweden 1979)

Squamous cell carcinoma (SCC) is the dominating histological type of primary malignant tumours in the head and neck region. These tumours are mainly localized to the lips, oral cavity, larynx, pharynx and nose/paranasal sinuses. In contrast, adenocarcinomas are the principal histological types of tumour in the salivary glands and the thyroid.

The squamous cell carcinomas are often in an advanced stage at diagnosis (Table II). Survival rates (Table III) and disease free intervals are low.

Table II. *Clinical stage of SCC at diagnosis in relation to site*

Location	Stage				Author(s)
	I	II	III	IV	
Tongue	9	12	35	34	Leipzig & Hokanson, 1982
Tongue (anterior 2/3)	51	146	111	5	Strong, 1979
Base of tongue	1	15	82	32	Strong, 1979
Oral cavity	115	60	95	532	Platz et al, 1982
Tonsils	22	102	118	111	Fayos & Morales, 1983
Max. sinuses	-	21	283	170	Sakai et al, 1981

Table III. *5-year relative* survival of patients with SCC in relation to site*

Location	Localized disease (%)	Regional metast. (%)	Distant metast. (%)	All stages (%)
Lip	89	57	-	86
Floor of mouth	65	31	18	45
Tongue	52	22	7	33
Oropharynx	44	23	11	28
Nasopharynx	44	25	10	26
Hypopharynx	20	14	12	15

(Management guidelines for head and neck cancer, 1979)

(*Defined as ratio of observed survival rate to rate expected of individuals in a general population with same age, sex, race and calendar year of observation.)

Management guidelines for advanced resectable SCC of the head and neck include pre- or postoperative radiotherapy and surgery. For unresectable tumours radiotherapy is given with a curative intention. Survival figures after such treatment are not acceptable and treatment has to be improved.

Improvement of therapy might be achieved in the following ways:

1. Extensive surgery with wider resection marginals combined with different reconstructive procedures such as myocutaneous flaps.

2. Improved radiotherapy, e.g.
   - hyperbar oxygen in combination with radiotherapy
   - fast neutrons
   - radiosensitizers of hypoxic cells
   - multiple doses per day
   - hyperthermia in combination with radiotherapy

3. Chemotherapy in combination with surgery and/or radiotherapy.

The role of chemotherapy in the treatment of SCC of the head and neck has been debated. However, different phase II trials have demonstrated a potential effect of this modality. From being regarded as a tumour unresponsive to chemotherapy it is now considered to be responsive, and palliative treatment has become curative with the introduction of new drugs and new combinations of drugs (Frei, 1982).

In the treatment of head and neck tumours anticancer drugs such as methotrexate, 5-fluorouracil, cisplatinum, bleomycin and mitomycin C have turned out to be active agents used both singly and combined (Glick et al, 1980).

Treatment schedules for combinations of anticancer drugs are usually based on clinical experience. However, more understanding concerning the nature of the tumour cells, untreated as well as influenced by surgery, radiation or chemotherapy, is important. This is also stated by Ervin et al (1981) who conclude that the design of combination chemotherapy for SCC of the head and neck must reflect experimental tumour systems as well as clinical experimental data.

In vitro systems with malignant cells of human or animal origin allow experimentation under well-defined conditions, but important factors such as tumour-host interaction, vascularisation, drug metabolism and distribution cannot be tested.

Experimental *in vivo* investigations can be undertaken on animal tumours, usually rapidly growing transplantable tumours in rodents. Such studies have provided wide knowledge on cell kinetics. These tumours, however, have biological

characteristics which differ from human malignancies. Most are anaplastic sarcomas, few are carcinomas and none are well differentiated (Rockwell, 1980).

The introduction of human tumours heterotransplanted into immune deficient animals has raised considerable interest since it has turned out that heterotransplanted tumours retain many human characteristics.

Human tumours were early successfully heterotransplanted to immunologically privileged sites, such as the anterior chamber of the eye of guinea pigs and rabbits (Greene, 1941), the brain of guinea pigs and mice (Greene, 1951) and the hamster cheek pouch (Lemon et al, 1952). However, in the latter system a hybridisation of human tumour and normal hamster cells was found (Goldenberg et al, 1974).

In 1966 it was shown that mice, after neonatal thymectomy, accepted heterografts of in vitro cultivated human tumour cells (Osoba & Auersperg, 1966). Thymectomy in combination with whole-body irradiation and marrow-reconstitution of mice, later resulted in a suitable system for heterotransplantation (Steel et al, 1978). In immune-suppressed mice there is however, in contrast to nude mice, some recovery of immunity, which constitutes a drawback in long-term experiments.

a. THE CELL CYCLE

From studies on the incorporation of radioactive phosphorous into the DNA of bean roots (Vicia faba) Howard and Pelc (1951, 1953) could deduce that the period between two mitoses could be divided into a period of DNA synthesis (S), preceded by a gap (G1) between the end of the first mitosis and the DNA synthesis, and followed by another gap (G2) between the end of DNA synthesis and the subsequent mitosis (Fig. 1). Before this was made known, cell kinetic studies had mainly been concerned with investigations of the growth rate or tumour volume doubling times (DT) of tumours (c.f. Tubiana, 1982).

More thorough cell kinetic studies of mammalian cells were made possible mainly by the introduction of tritiated thymidine. Tritiated thymidine allowed labelling of cellular DNA and the kinetics of normal and malignant cells in vivo and in vitro could be studied.

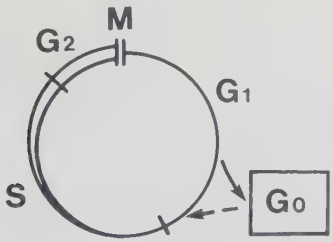

Fig. 1. The cell cycle phases.

In the cell cycle, reviewed by Baserga (1981), proliferating cells go through various phases in their progress from one mitosis to the next. After mitosis the cells can either leave the cell cycle and enter the G0 phase or continue into the G1 phase of the next cell cycle. G0 cells can either die or re-enter the cell cycle.

Cell cycle time is often defined as the interval between the midpoint of two subsequent mitoses. In the cell cycle the time of the G1 phase is generally the most variable and its length largely influences the rate of cell proliferation. From studies of normal human tissue and tumours in man it is also clear that tumour cells compared to normal cells, proliferate slower rather than faster (Tannock, 1978).

Growth of normal tissue and malignant tumours is mainly caused by an increase in the number of cells and/or an increment in the size of individual cells. Occasionally volume changes may result from oedema, necrosis, suspension of cell renewal etc. Such processes are second in importance to increcrement of cells through cell division (Stragand et al, 1982).

Solid tumours are not homogeneous, exponentially growing masses of proliferating cells. The tumour cells fall into

three categories of compartments (Fig. 2) according to Mendelsohn & Dethlefsen (1973): proliferating cells (P), non--proliferating cells (Q) and cells that are lost due to cell death or migration (L).

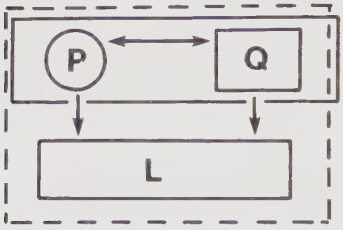

Fig. 2. Model of tumour growth with P (proliferating), Q (quiescent) and L (lethal) compartments, flow of cells and actual and virtual volumes.

P cells are the only cells that contribute to tumour growth and the net effect depends on the duration of their cell cycle and their conversion to Q and L cells. The actual tumour volume is made up by the cells in P and Q compartments. The proportion of proliferating cells or growth fraction (GF) is defined as P/P+Q. The effect of cell loss can be imagined in terms of virtual volume which contains the actual cellular volume plus dead, dying and otherwise departed L cells. The virtual volume is the volume that would include the already departed L cells. It thus represents the potential growth of the tumour. There is a corresponding total volume which includes the acellular contents of the tumour (e.g. necrotic regions, cysts, alveolar and ductal spaces and vascular space).

Tumour cell kinetic studies can be conducted in man or in experimental systems. Studies in man require multiple sequential biopsy specimens and often administration of radioactive labelled precursors. Besides ethical problems it is usually technically difficult to undertake such investigations and data on SCC of the head and neck are sparse (Frindel et al, 1968; Bresciani et al, 1974).

b. THE NUDE MOUSE

The mouse mutant "nude" (nu/nu) was first reported in 1962 (Isaacson & Cattanach, 1962), Though it was thoroughly described by Flanagan in 1966, the absence of thymus was not detected until 1968 (Pantelouris, 1968). As a consequence of the athymic state thoracic duct lymphocytes from nude mice lack T-lymphocytes and compromise a virtually pure population of B-lymphocytes (Sprent, 1974). Although they lack mature T-lymphocytes, adult nude mice have higher levels of NK (natural killer cell) activity than do most conventional mice (Holden et al, 1978). Despite the lack of T-lymphocytes, nude mice can synthetize IgG1 and IgG2, though the levels are low. IgM levels are usually normal (Okudaira et al, 1977). The complement, as in conventional mice, seems to be very innefficient (Koene et al, 1974). The congenital absence of the thymus and the impaired cell mediated immune response makes heterotransplantation of tissue possible.

BREEDING AND MAINTENANCE

The impaired immune response makes the nude mouse susceptible to infection and it has a relatively short life span in a conventional environment. Longevity increases with the degree of "cleanness" of the environment (Eaton et al, 1975; Ovejera et al, 1978), and under germ-free conditions, as in germ-free isolators, nude mice have the same life expectancy as conventional mice. The major risk for microbial contamination originates from man. However, it is possible to produce large numbers of nude mice in a modified conventional environment, provided that precautionary techniques such as filter covered sterile cages, laboratory uniforms, masks and gloves are used (Poiley et al, 1974). In the production of nude mice the breeding scheme is important, since the female nude mice are poor nurses. The optimal scheme is a male nude homozygote (nu/nu), mated to a female heterozygote (nu/+) (Giovanella & Stehlin, 1973). With this scheme half of the progeny are nudes and half heterozygotes, which can be used for further breeding.

The condition of the animals is of importance, since caloric restriction weight loss (Giovanella & Shepard, 1982) as well as mouse hepatitis virus (Kyriazis et al, 1979) and other infections suppress tumour growth.

HETEROTRANSPLANTATION OF HUMAN TUMOURS TO NUDE MICE

The first successful heterotransplantation to nude mice of a human malignant tumour, a rather highly differentiated mucoid--producing adenocarcinoma of the sigmoid colon, was reported by Rygaard & Povlsen in 1969. Since then a number of histologically different malignant tumours have successfully been established as tumour lines on nude mice (see: List of human tumours transplanted to nude mice, 1977).

During serial transplantation the heterografted tumours retain their human tumour characteristics such as chromosome pattern, isoenzyme pattern, antigenicity and histology (Povlsen et al, 1973 b; Povlsen et al, 1975). However, in the growing heterotransplanted tumours only the tumour cells are of human origin, vascular and connective tissue are derived from the host (Warenius, 1980).

Heterotransplantations are only successful in about one third of the cases, and only about two thirds of the accepted grafts can be serially transferred into new nude recipients (cf. Povlsen, 1980). The success rate differs between tumour types; malignant melanoma, colon, skin and lung tumours are easy to establish whereas endocrine, hormone dependent tumours and malignancies of the lymphoid tissue are very difficult (Reid et al, 1978; Povlsen, 1980). Benign tumours may grow in the first passage but fail in subsequent transplantations (Reid et al, 1978; Fogh et al, 1980). It is also shown that metastatic and recurrent tumours will grow more readily than will primary tumours (Fogh et al, 1980).

In a direct comparison of the parameters of the Gompertz equation (Winsor, 1932; Akanuma, 1978), when fitted to the growth curves of the lung metastases of a testis cancer and the growing heterotransplants from the metastases, there was no significant difference. However, the tumour transplants had approximately ten times faster initial growth and 20 times faster retardation of growth than the pulmonary metastases (v Eyben et al, 1982).

FACTORS AFFECTING GROWTH AND METASTASES OF HETEROTRANSPLANTED TUMOURS

It was early recognized that though the human tumours that were heterotransplanted exhibited malignant criteria such as invasive growth and metastasis in man, infiltration was not seen in s.c. inoculated surgical specimens growing on nude mice. In the first reports the tumours rather seemed to grow as well-circumscribed, apparently encapsulated, solid masses on section. Microscopically the tumours appeared circumscript, but not encapsulated (Povlsen & Rygaard, 1971). This was later shown in a study of 122 successfully transplanted tumours where invasion was very infrequently seen (Sharkey et al, 1978).

The prevalence of metastases from heterotransplanted tumours differs. Initially metastatic growth in nude mice heterotransplanted with solid tumour specimens was not seen (Povlsen & Rygaard, 1971; Povlsen & Rygaard, 1972, Povlsen et al, 1973 b, Sordat et al, 1974). Metastasis was first reported by Giovanella et al (1973, 1974) who injected human derived cultured cell lines intradermally or s.c. and in some cases detected metastasis to lymph nodes and lung. Metastases after injection of cultured cell lines have also been observed by other authors (Pettengill et al, 1980; Tropé et al, 1982).

Metastases are occasionally also observed after s.c. inoculation of surgical tumour specimens, though this is rare (1.3%) (Sharkey & Fogh, 1979). Tumours derived from cultured cell lines seem more prone to metastasis than those derived from surgical specimens. There is an association between metastasis and body-wall invasion (Sharkey & Fogh, 1979).

Furthermore, the barrier against metastasis can be overcome by using neonatal nude mice. Sordat et al (1977) demonstrated that tumour lines normally not metastasizing in nude mice, disseminated when inoculated in neonatal nude mice. The ability of the tumours to disseminate dropped rapidly within a few days after birth.

Metastasis can also be induced in nude mice from normally non-metastatic tumours by sublethal irradiation of the mice or treatment with anti-lymphocyte serum (Klein et al, 1978), which suggests that the suppression of metastasis is of cellular origin. Metastasis can also be promoted by administration of normal human T-lymphocytes (Graham et al, 1978).

Macrophages are possibly also involved in the suppression of tumour growth, since it has been demonstrated that Carragheenan, a macrophage-toxic agent, increases the take--rate of serially transplanted heterografts (Kopper et al, 1980). However, not only the cell mediated immunity seems to be involved in rejection. Koene et al (1974) showed that nude mice with skin allografts, were able to reject these if they were given rabbit complement. Mouse native complement is very weak and nude mouse antibodies were able to destroy grafts if efficient complement was added to the system. It is clear that nude mice are capable to form antibodies directed against transplantation antigens.

A further factor influencing growth of heterotransplants is the background strain of the nude mice (Maruo et al, 1982) and the site of inoculation on the nude mouse lateral trunk (DiPersio, 1981), where the anterior position was superior to the posterior.

CHEMOTHERAPY OF HETEROTRANSPLANTED TUMOURS

The usefulnes of human tumours heterotransplanted to nude mice as a model for experimental chemotherapeutic studies was soon recognised, and in 1972 it was demonstrated that heterotransplanted tumours responded to BLM and CCNU (Povlsen et al, 1973 a). Since then many reports on chemotherapeutical studies in nude mice have been published (c.f. Giovanella, 1980). In most reports changes in tumour volume or weight have been the end-points of the studies.

The drug doses applied to man cannot be used in nude mice experimentation. Drugs are less toxic in conventional mice than in man (Giovanella & Stehlin, 1980), and nude mice

tolerate even higher doses than conventional mice (Houchens et al, 1977). This can be attributed to a more active microsomal system in the liver and kidneys of nude mice (Freudenthal et al, 1976; Litterst et al, 1978). Toxicity data concerning nude mice for several commonly used drugs have been published by Houchens et al (1977) and Povlsen (1978).

The rationale for using heterotransplanted human tumours instead of transplantable rodent tumours for chemotherapeutic studies, is the assumption that heterotransplanted tumours retain their sensitivity to chemotherapeutic drugs. The relevance of this assumption is supported by findings that sensitivity of heterotransplanted tumours to commonly used drugs correlates fairly well to the clinical experience of the histologically same type of tumours (Giovanella et al, 1978; Giovanella et al, 1983). There is also accumulating evidence on similar response in direct patient - heterotransplant comparisons (Giovanella et al, 1978; Fodstad et al, 1980; Fujita et al, 1980; Shorthouse et al, 1980).

However, within the same histological type of tumours, sensitivity differs between individual tumour lines, as shown for malignant melanoma by Bellet et al (1979). Since chemotherapeutic drugs are not efficient against all tumours of the same histological type, the use of one single heterotransplanted tumour line for preclinical screening is precluded. The problem is discussed by Bellet et al (1979), who suggest the use of a tumour panel for this purpose.

β_2-MICROGLOBULIN IN HETEROTRANSPLANTED TUMOURS

β_2-microglobulin (β_2m) is a small polypeptide, related to the immunoglobulins and present on nucleated cells as part of the strong transplantation antigens (c.f. Berggård et al, 1980). It is present in normal human tissues and various biological fluids. Raised plasma levels have been recorded in patients with various malignant diseases (Shuster et al, 1976). Human β_2m has been immunohistochemically detected in carcinomas on nude mice, established from in vitro cultivated tumour lines (Michael et al, 1980), and in the plasma of tumour bearing mice (Michael et al, 1980; Biörklund et al, 1980).

Aims of the Study

The nude mouse has been used for chemotherapeutic investigations for ten years. Though there are obvious differences in for instance tumour volume doubling time and maximal achievable volume between heterotransplants and corresponding tumours in man, little is known about changes in growth and tumour cell kinetics induced by the heterotransplantation itself. Furthermore, most chemotherapeutic studies have only focused on changes induced in tumour volume growth. It was therefore considered of interest to study the course of cell kinetic events in the first passages after heterotransplantation and after drug treatment of animals with established tumour lines. The aims of the study have thus been:

- to investigate to what degree malignant head and neck tumours, especially SCC, can be heterotransplanted into nude mice, and particulary to study the influence of sterility on the take rate of transplanted tumour specimens.

- to investigate the influence of the degree of histopathological differentiation, stage of donor tumour disease and DNA synthesis on take rate.

- to investigate and define changes in growth and some cell kinetic parameters at heterotransplantation and in the first passages of heterotransplanted tumours in nude mice.

- to study the occurrence of human β_2m in heterotransplanted tumour lines and in the plasma of tumour bearing mice, and to compare data with the presence of β_2m in patients with SCC of the head and neck.

- to treat heterotransplanted tumours with cytostatic drugs and study their effect on tumour growth and some cell kinetic parameters.

Materials and Methods

NUDE MICE

For heterotransplantation five to eight week-old male and female BALB/c nude (nu/nu) mice were used. Heterozygous mice were first supplied by Gl.Bomholtgaard Ltd, Ry, Denmark, and later bred in our laboratory by mating heterozygous (nu/+) females with homozygous (nu/nu) males. The colony was kept under sterile but not specific pathogen-free conditions. The mice were held in sterile cages under filter tops and with coarse sawdust bedding. They were fed on sterilized ordinary laboratory food and acidified (pH 2.5-2.7) water ad libitum. Antibiotics were not given. The ambient temperature was 25-26°C.

Transplantation of tumour specimens was done under aseptic conditions directly after excision of heterotransplants or, in cases of biopsies from patient tumours, no later than three hours after excision. The animals were anesthetized with chloralhydrate i.p. (0.5 mg/g b.wt.). The tumour specimens were cut into small pieces measuring 2x2x2 mm before transplantation and each graft was inoculated separately s.c. on the side of the back of the mice. In some cases mice with two tumours, one on each side of the back, were used. This metod reduces the number of animals needed for the experiments, and we have in accordance with the results of Warenius et al (1980) and Spang-Thomsen et al (1980) not found any host induced uniformity in growth of importance.

DRUGS

All drugs used for dose-response and cell kinetic experiments were obtained as commercially available preparations. Before administration they were diluted to proper concentration and administered in volumes of 0.01 - 0.02 ml/g b.wt.

In the evaluation of each drug the dose-response relationship was studied. The doses used in the subsequent cell kinetic studies were based on the dose-response curves.

MITOMYCIN C. The mitomycins are antibiotics with antitumour, antibacterial and antiviral activities. The first isolation was from broth cultures of Streptomyces caespitosus by Hata (Hata et al, 1956). Mitomycin C was isolated and purified in 1958 (Wakaki et al, 1958). It is an alkylating agent and has three potentially active groups. MMC induces inter- and intrastrand crosslinks in DNA (Iyer & Szybalski, 1964), can degrade DNA (Lown & Weir, 1978) and inhibit DNA synthesis

(Schwartz et al, 1963). Clinically MMC have an established effect against SCC of the head and neck (Graham et al, 1967; Inuyama, 1979).

CISPLATIN. Cisplatinum (CDDP; cis-diamminedichloroplatinum II) is a square planar, heavy metal complex containing a central platinum atom surrounded by two chloride atoms and two ammonia molecules in cis position. CDDP is bifunctional in that each molecule has two sites for reaction (Zwelling & Kohn, 1980). CDDP was discovered in 1965 as a compound with antibiotic activity (Rosenberg et al, 1965). Subsequent studies (Rosenberg et al, 1969) established the antitumour activity of CDDP in experimental animal tumours.

The effects of CDDP on DNA have recently been reviewed by Zwelling & Kohn (1980). CDDP has been shown in mammalian cells to concentrate in the nucleus, bind to DNA and inhibit DNA synthesis. Its alkylating properties allow bifunctional linking to guanine bases, the formation of intrastrand crosslinks between guanines and formation of interstrand crosslinks of DNA.

Clinically CDDP has a well established efficacy against SCC of the head and neck (Glick et al, 1980; Mead & Jacobs, 1982).

BLEOMYCIN. BLM is a complex glycoprotein antibiotic, originally isolated from Streptomyces verticillus by Umezava et al (1966). BLM binds to DNA (Umezava, 1974). This binding is thought to be a partial intercalation in the major groove. After binding, BLM excises free bases, resulting in single strand breaks. Double strand scission occurs at higher concentrations of BLM, and is thought to take place when there are enough single strand breaks to result in a double strand break (Saunders et al, 1975).

Clinically, BLM has among other malignancies an established efficacy against SCC and plays a major role in the treatment of SCC of the head and neck with combination chemotherapy (Glick et al, 1980).

TUMOUR VOLUME MEASUREMENTS

Two perpendicular diameters of the tumour were measured with graduated calipers. The tumour volume was then calculated according to the formula for a rotating elipsoid:

$$\text{Volume} = \frac{\text{length} \times \text{width}^2}{2}$$

Tumour volume calculated according to the formula correlates well with tumour weight (Osieka et al, 1977; Fodstad et al, 1980). Tumour diameters were measured at regular time intervals.

In paper IV chemotherapeutically induced growth retardation was quantified as Relative Tumour Size - RTS - (1 week), i.e. the tumour volume one week after drug administration in relation to the tumour volume at the time of drug injection (Lesser et al, 1980). The formula is:

$$RTS = \frac{\text{Volume } (t = 1 \text{ week})}{\text{Volume } (t = 0)}$$

RTS-values were calculated for the different dose levels and transformed employing the square root of RTS in order to achieve normality (Lesser et al, 1980). Dose-response curves then could be constructed.

In paper V a modified model for estimation of chemotherapeutically induced growth retardation was used. At regular time intervals Relative Tumour Size (RTS) i.e. the tumour volume after drug administration in relation to the tumour volume at the time of the first drug injection was calculated. The formula is:

$$RTS = \frac{\text{Volume } (t = \text{day } n)}{\text{Volume } (t = 0)}$$

The data were transformed employing the square root of RTS, in order to achieve normality. For each tumour the data were displayed as the graph of the square root of RTS versus time, and the area under the graph was taken as a measure of tumour growth, as described by Lesser et al (1980).

MEASUREMENT OF DNA-SYNTHESIS

The incorporation of tritiated thymidine (3HTdR) into tumour DNA was measured as an estimate of the rate of DNA synthesis using the technique described by Håkansson & Tropé (1973). The solid tumour was cleaned from necrotic debris and converted to a suspension by pressing it through a stainless steel mesh. The cell suspension was incubated at 37°C for one hour with 3HTdR (spec. act. 1.9 Ci/mM) at a final concentration of 2 mCi/ml. It was then washed with phosphate buffer,

and the cells were precipitated with cold trichloroacetic acid to extract nucleosides and nucleotides. The amount of 3HTdR incorporated into DNA and the total amount of DNA in each sample was measured. All tests were performed twice.

By relating the incorporation of labelled thymidine, measured as CPM, to the total amount of DNA in the sample, it is possible to compare samples containing different numbers of tumour cells. The amount of incorporated 3HTdR per unit DNA in the sample is expressed as a logarithmic function (Håkansson & Tropé, 1973). The formula is:

$$\underline{a} = 100 \times {}^{10}\log \frac{\text{CPM} \times \underline{k}}{\text{DNA}}$$

CPM is the number of counts per minute registered. Counting is done for 5 minutes in a Beckman Liquid scintillator 7000. $\underline{k}$ is a factor needed to correct for i.a. counting efficiency. $\overline{\text{DNA}}$ is the amount of DNA in the sample determined with the method of Bonting & Jones (1957) and expressed in arbitrary units.

The thymidine uptake in a cell sample, as determined by the above expression, gives near-normal distributed variates.

MEASUREMENTS OF DNA CONTENT IN INDIVIDUAL CELL NUCLEI

The basic principles and methods of flow systems for analysis of mammalian cells have been reviewed by Crissman et al (1975). In the present study tumour samples for flow cytometric (FCM) analysis were kept frozen at -70°C in dimethylsulfoxide/citrate buffer. For the preparation of nuclei for flow cytometric DNA analysis the tumour pieces were thawed and immediately converted into a single cell suspension. They were minced with sharp scissors and mashed through a nylon net (pore size 140 µ). Tris-buffer (pH 7.5) was continuously poured over the net during the procedure. The tumour-cell suspensions were centrifuged for 15 minutes at 250 g at 20°C. The cells were fixed in absolute alcohol at -20°C. Fixed cells were exposed to 0.1% RNase (Sigma R5125, dissolved in 5.5% of 1 N HCl) for 5 minutes at 20°C. The cells were centrifuged and treated with 0.5% pepsin (Merck) for 10 minutes at 37°C, after which they were cooled, centrifuged and resuspended in Tris-buffer to a concentration of about 10^6 cells per ml. One ml of the suspension was stained with 0.005% propidium iodide (Calbiochem) (Krishan, 1975) and 0.6% Nonidet (Merck) dissolved in Tris-buffer. Before flow cytometric analysis in a Cytofluorograf System 50-H (Ortho Instruments, USA) the stained cells were filtered through a 50 µ nylon net. An argon laser was used for excitation at 488 nm by 450 mW. Doublets of cells were excluded by electronical threshold settings. Ten to 100 thousand cells were

counted in each individual sample.

The DNA content of mouse lymphocytes was reported to be 82% of the DNA content of human lymphocytes (Handbook of Biochemistry and Molecular Biology, 1976), which is in accordance with the findings in the present study (IV). Thus it is possible to separate normal mouse cells from human diploid cells.

IMMUNOFLUORESCENT STAINING OF TISSUE β_2M

For immunohistochemical identification of tissue β_2m tissue specimens were frozen in isopentane quenched to the temperature of liquid nitrogen, in which they were stored. For further processing by the indirect immunofluorescence method of Coons (1958) cryostate sections were cut at a thickness of 8 μ, melted to glass slides and air dried. They were incubated for 30 minutes at room temperature in rabbit-antihuman β_2m diluted in phosphate buffered saline (PBS) with 5% normal swine serum (NSS) to titres of 1/20, 1/40, 1/80, 1/160 and 1/320. After washing in PBS for 5 minutes the slides were incubated for 30 minutes at room temperature in fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulin diluted to 1/20 in PBS with 5% NSS. After washing in PBS for 5 minutes the slides were mounted in PBS with 10% glycerol. As controls with known positive reactivity slides with sections of skin were used (c.f. Malmnäs Tjernlund & Forsum 1977). For negative controls rabbit antihuman immunoglobulin was used in the first incubation step or the last incubation step with the FITC-conjugated antiserum was omitted. (All antisera and normal swine serum were purchased from DAKO Immunoglobulins AB, Stockholm, Sweden.)

DETERMINATION OF PLASMA AND TISSUE β_2M CONCENTRATION

Plasma sample collection: Plasma samples from patients were obtained by cubital vein puncture. Blood was collected in heparinized syringes, centrifuged at 800 g for 10 minutes. The supernatant was stored at -20°C until analysis. Blood from nude mice was obtained through cardiac puncture and then treated as described above.

Extraction of β_2m from solid tumour: The tumour specimen was weighed and then minced with scissors in 0.9% NaCl. The suspension was homogenized using an ice bath (Potter-Elvehjem homogenizer). The homogenate was stored at -70°C until analysis. It was then thawed and centrifuged at 105.000 g for one hour. The supernatant was collected and diluted. β_2m concentration was determined as described below.

Radioimmunoassay for human β_2-microglobulin: Human β_2m was purified from urine by a modification (Berggård et al, 1980) of the original procedure (Berggård & Bearn, 1968). The protein was radiolabelled by the Chloramin T method (Greenwood et al, 1963). It was then used in a radioimmunoassay as

described by Plesner et al (1975) together with a goat antihuman β_2m antiserum, produced as reported by Björck et al (1977). Immune-complexes were separated from unbound ^{125}I-human β_2m by polyethylenglycol-exclusion (PEG 6000, Kebo AB, Stockholm, Sweden). The β_2m concentrations were determined at final dilutions of 1:250 (human plasma) or 1:25 (mouse plasma). At such dilutions, the mouse β_2m of mouse plasma did not interfere with the assay. The interassay variation, as estimated by multiple determinations on selected plasma samples, was approximately 10%. The upper limit of normality (mean + 2 S.D.) in man was defined from a control material previously reported (Tropé et al, 1980).

The Present Investigations

HETEROTRANSPLANTATION OF MALIGNANT HEAD AND NECK TUMOURS (I)

An in vivo system for tumour cell kinetic studies, the nude mouse system, was evaluated for transplantation of human malignant head and neck tumours. A total of 54 human malignant tumours of the head and neck, 46 squamous cell carcinomas and 8 other malignant tumours, were transplanted to nude mice.

Results: Nineteen of the tumours (35%) grew as localized tumours in one or more transplantation sites. There was no difference in take rate between heterotransplants from 38 "non-sterile" dignostic biopsies and 16 "sterile" per-operative specimens. Ten out of the 19 transplants that grew in the first passage, resulted in established tumour lines, i.e. tumours could be passed through three or more subsequent transplantations to nude mice.

There was no difference in take rate between squamous cell carcinomas with different degrees of histopathological differentiation ($p>0.05$).

Forty-five of the 46 transplanted SCC were staged according to the UICC classification. The take rate for T4 and clinical stage IV tumours seemed to be higher than for less advanced tumours ($0.1>p>0.05$).

Tumour material from 13 transplanted tumours could be analysed in vitro with respect to 3HTdR incorporation into DNA. There was no difference between biopsy and peroperative specimens nor between specimens that were rejected or grew as heterotransplants. Six tumours that resulted in tumour lines could be analysed with respect to 3HTdR incorporation into DNA in their first, second and fifth passage in nude mice. In the first passage the rate of 3HTdR incorporation was in the same order as for the thirteen transplanted tumours. In the second and fifth passage the 3HTdR incorporation was significantly increased.

Conclusion: The low frequency of resulting tumour lines (10/54 = 19%), and the considerable time needed to establish a tumour line makes the nude mouse system unsuitable for a predictive test system for e.g. chemosensitivity testing. Though there was no difference in 3HTdR incorporation between tumours that were accepted or rejected, a probable higher take rate for advanced tumours makes it reasonable to believe that intrinsic properties are of importance for acceptance and survival of the heterotransplanted tumour. The increase in 3HTdR incorporation during the first passages in nude mice,

might be explained by mechanisms such as recruitment of resting G0 cells, increase of growth fraction, selection of rapidly proliferating cells or a decrease in cell loss factor.

CHANGES IN GROWTH AND CLONAL COMPOSITION OF HETEROTRANSPLANTED TUMOURS (II)

Six human squamous cell carcinomas of the head and neck heterotransplanted to nude mice were studied with respect to changes in tumour volume doubling time (DT), cell cycle phase distribution and clonal composition during serial passages.

Results: The tumour lines exhibited a statistically significant decrease in DT between the first and third to the fourth passage towards a limiting value. There was also with increasing passage a tendency towards a shortening of the latency period between transplantation and observable growth.

Five of the original tumours and serially passed heterotransplants were analysed with flow cytometry. Two tumours were diploid and remained so after heterotransplantation. Three tumours had one diploid and one aneuploid cell population. At heterotransplantation there was a selection of clones and only the aneuploid cell populations could be recovered from the heterotransplants in the first as well as in later passages.

Conclusions: Since DT only reflects the net growth rate, the observed decrease in DTs of the tumour lines can be explained by either changes in growth fraction, Tc or cell loss factor. Previously reported results (Pickard et al, 1975; Houghton & Taylor, 1978) makes it reasonable to attribute the changes in DT to a decrease in cell loss.

The selection of clones at heterotransplantation might mirror differences between clones in biological aggressiveness and intrinsic capacity to overcome host resistance.

β_2-MICROGLOBULIN IN SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK AND IN HETEROTRANSPLANTED TUMOURS (III)

The occurrence of human β_2m in plasma was studied in 26 patients with squamous cell carcinoma of the head and neck and in 6 heterotransplanted carcinoma lines. Extractable β_2m was measured in biopsies from 7 patients with SCC of the head and neck and in the 6 heterotransplanted tumour lines. Immunohistochemically detectable β_2m was studied in 20 patient tumours and in all heterotransplanted tumour lines.

Results: Stage IV tumours seemed to have higher p-β_2m, than less advanced tumours. However, only three of 26 patients (11.5%) had p-β_2m values above the upper level of normality. There was no relation between the calculated total amount of extractable β_2m in the patient tumours and p-β_2m. However, there was a probable association between the concentration of β_2m in the seven studied tumours and p-β_2m ($0.10>p>0.05$).

Human β_2m could be detected in the plasma of mice with tumours from all tumour lines and there was an association between tumour volume and p-β_2m. The ratio p-β_2m/tumour volume, differed between the tumour lines. Tumour volume doubling time (DT) was determined for the different tumour lines and there was a correlation ($0.05>p>0.02$) between DT and β_2m concentration of the tumours. The mean concentration of extractable β_2m was ten times higher in patient tumours than in heterotransplanted tumours. Membrane bound human β_2m was immunohistochemically detectable both in SCC of the head and neck and heterotransplanted tumours. The observed distribution was smooth, gracile and linear in normal epithelium and smooth to granular and irregular in tumour specimens.

Conclusion: The low rate of pathologically raised p-β_2m makes it unsuited as a tumour marker for detection of SCC. It might, however, in the individual patient be of interest as a complement to physical examination, in monitoring and follow-up of treatment. The higher β_2m concentration in patient tumours compared to heterotransplants can, since there is a correlation between DT and β_2m concentration, be explained by faster growth of heterotransplants. Determination of β_2m concentration in patient tumours might also be used to assess the rate of tumour growth.

CHEMOTHERAPEUTIC INDUCED CHANGES IN GROWTH AND CELL KINETICS OF HETEROTRANSPLANTED TUMOURS (IV; V)

A poorly differentiated squamous cell carcinoma of the head and neck heterotransplanted to and established as a tumour line in nude mice was used to study chemotherapeutically induced cell cycle perturbations. The heterotransplanted tumour was treated either with mitomycin C or cisplatin administered i.p. in a single dose, or BLM i.p. once daily for four consecutive days. After determination of dose--response relationships and toxicity, treated tumours were biopsied at different intervals and cell cycle distribution pattern, and tumour volume was recorded. In the CDDP and MMC treated tumours 3HTdR incorporation into DNA and histology was also followed.

Results: All drugs gave highly significant dose-dependent retardations of tumour growth. However, with acceptable toxic doses it was not possible to achieve any reduction of tumour volume. The dose-response curve for BLM was concave and exhibited a plateau-phase beginning at a total dose of 200 mg BLM/kg.

Mitomycin C and cisplatin gave the same pattern of cell cycle perturbations, although the changes induced by cisplatin were more profound. There was an initial, transient increase of the fraction of cells in S phase, concomitant with a reduction of the fraction of cells in G0+G1 phase. When these perturbations were normalized a transient increase of the fraction of cells in G2+M phase was observed. While cisplatin caused an initial transient depression of DNA synthesis, the mitomycin C treated tumours exhibited a short-lasting increase of DNA synthesis which preceded the increase of the fraction of cells in S phase. The maxima of the induced changes in cell cycle phase distribution and DNA-synthesis lasted only for 24-48 hours.

A single dose of 75 mg BLM/kg caused a transient accumulation of cells in G0+G1 phase and seemed to be followed by a synchronized passage of cells through the cell cycle. However, the induced perturbations were small. Iterated administration of BLM did not substantially increase the induced perturbations.

Conclusions: The sequence of events after CDDP and MMC administration can be interpreted as a reversible block in the transition of cells between S and G2+M phase resulting in a transient accumulation of cells in S phase and followed by an increase in the fraction of G2+M cells. The increase in DNA synthesis preceding the rise of the fraction of S phase cells in the MMC treated tumours might represent increased DNA repair after MMC inflicted damage.

Though both MMC and CDDP induced profound changes in tumour volume growth and cell kinetics, there was no change in the

histopathological picture of the treated tumours. Routine histopathological examination is thus not likely to be of value for the evaluation of the effect of chemotherapy with MMC or CDDP.

BLM is often used in combination with other drugs in phase-specific schedules, since it, apart from its moderate effect on the bone marrow, has been considered as a synchronizing agent. However, the reported effects on the cell cycle are contradictory, and the present study could not confirm any major cell synchronizing effect of BLM on heterotransplanted human squamous cell carcinoma.

General Discussion

METHODOLOGICAL CONSIDERATIONS

Tumour measurements and end-points: The method of tumour measurement and volume calculation does not take the third dimension - height - or skin thickness in consideration. However, there is a satisfactory correlation between calculated volume and weight of the tumours with a correlation coefficient (r) ranging between 0.918 and 0.994, dependent on the tumour line. To exclude interobserver variations, all measurements in each individual experiment were done by one person.

The same end-points are available for heterografts as for experimental syngenic animal tumours: growth delay, cell survival, inhibition of isotope incorporation and tumour control (Begg, 1983). Cure and survival, as discussed by Steel & Peckham (1980), are uncertain end-points since host defence mechanisms can be expected to modify the result. Growth delay is a common measure of therapeutically induced effects. There are however drawbacks with this method (Begg, 1980). Other authors recommend Relative Tumour Size - RTS -, and assuming exponential growth during the experiment, there is also a relation, which can be mathematically expressed, between growth delay and the difference between the RTS of treated and control tumours (Warenius et al, 1980).

There is an obvious risk that the RTS-values are not normally distributed, and not suitable for analysis with parametric tests. It is reasonable to transform the data by using the square root or logarithm of RTS (Lesser et al, 1980).

RTS has the advantage of enabling calculation of the area under the growth curve and allows comparison between non--exponentially growing tumours, taking both degree and duration of the induced tumour regression into consideration. Logarithmic transformation gives less weight to the degree of tumour regression and more to its time duration, with the square root behaving conversely (Lesser et al, 1980). In the present investigations the square root was used for transformation of data in order to achieve normality.

Measurement of DNA-synthesis: When 3HTdR incorporation is used to compare the DNA synthesis between untreated tumours, e.g. biopsies from tumours in man, and heterotransplanted tumours, it is important to bear in mind that the a-value (amount of 3HTdR incorporated per unit DNA) is an expression of the amount of incorporated labelled thymidine in relation to the total amount of DNA in the sample. It is thus important

that tumour specimens are cleaned from necrotic debris that can disturb the determinations.

Variations in thymidine incorporations might not always reflect changes in DNA synthesis. Nucleotide pools might change in size and salvage enzymes can be inhibited and even stimulated by administered drugs. Such changes can result in alterations in thymidine uptake without any corresponding change in DNA synthesis. The problem has been discussed by Håkansson (1975), who investigated the effect of cytostatic drugs on incorporation of labelled precursors into DNA and phosphorylation of thymidine in mouse thymocytes and chick embryo cells. All tested drugs caused a marked depression of incorporation of labelled precursors, but had no influence on the phosphorylation of thymidine. Since stimulation of the synthesis of a precursor pool is an unlikely action of a cytostatic drug, he concluded that changes in a-values were likely to actually reflect changes in synthesis of nucleic acids.

Flow cytometry: The parameters actually determined in flow cytometry are the area under and height of the light pulse emitted from propidium iodide (PI) (Krishan, 1975) after excitation. The aim of the preparation procedure is thus a pure suspension of single tumour nuclei, which can then be stained with PI. PI binds by intercalation with double stranded DNA.

Treatment with pepsin and the non ionic detergent Nonidet disrupts the cytoplasmatic membrane and results in nuclei practically devoid of cytoplasma (Vindelöv, 1977). Digestion with RNase is important for the elimination of double--stranded RNA, a potential binding source for PI, and thus interference with DNA determination (Krishan, 1975).

It is important to adjust the nuclear concentration before staining, since excess of nuclei (DNA) can reduce the amount of dye bound to DNA and thus also the intensity of the evoked fluorescence. This is specially important in determination of ploidy, when an external DNA standard, usually human lymphocytes, is used. Heterotransplanted tumours have the advantage of mouse derived stromal cells as an internal standard.

The mechanical treatment with repeated washing and filtering induces a risk of a skewness in the distribution of subsets of cells caused by differences in cell loss by filtration and adhesion to tubes, and thus also a bias in the cell cycle phase analysis. However, if there is such a selection, the induced fault is systematic and thus not likely to alter conclusions based on changes in cell cycle phase distribution in sequential samples.

In the evaluation of the DNA histograms (IV) the compound DNA histogram was regarded as made up by individual DNA distribution curves for the G0+G1, S and G2+M cell populations.

The G0+G1 and G2+M populations were estimated from the outer areas of each peak, and the S population was calculated on the basis of those estimations and the total amount of cells in the population. This is likely to result in a slight, systematic overestimation of the G0+G1 and G2+M populations and an underestimation of the S cell population since the areas for G0+G1 and G2+M determination are affected by the presence of S phase cells (Fried, 1976).

Another potential fault in the determination of cell cycle phase distributions is debris in the sample which can change the shape of the DNA histogram. However, the applied preparation method, when used on heterotransplanted SCC and SCC tumour biopsies, resulted in very small amounts of debris, usually located outside the region of interest and not interfering with the determination of cell cycle phase distribution.

CELL KINETIC CONSIDERATIONS

Though the nude mouse system has gained widespread popularity as a model for chemotherapeutic studies, investigations on changes in tumour volume growth and tumour cell kinetics induced by the heterotransplantation are sparse. This is remarkable as, for instance, differences in DT and maximally achievable volume between heterotransplants and tumours in situ are obvious. If conclusions based on results from this model are to be used for clinical considerations, it is of importance to define differences and similarities in growth and cell kinetics.

During the first transplantation of a tumour to the nude mouse, and its initial subsequent passages in the animal the transplant is the object for the remaining host resistance of the athymic nude mouse. The transplant either overcomes this resistance and adapts to the new environment or fails to grow.

Only 20 - 25% of transplanted malignant human tumours result in established lines, i.e. survive the first three passages in nude mice (Lindenberger et al, 1978; Fogh et al, 1980), which is in accordance with the result of the present study (I). Clinically advanced tumours (I) as well as metastatic and locally recurrent tumours (Fogh et al, 1980) seem to be more prone to "take" than less advanced and primary tumours. This might reflect differences in biological aggressiveness. "Take" as a prognostic factor has been demonstrated by Greene (1952). He found an inverse relationship between take of tumours transplanted to the anterior chamber of the eye of guinea pigs, and survival of the patients.

Heterotransplantation also results in a selection of aneuploid tumour cell clones (II) in that only the aneuploid cell population could be recovered in growing heterotransplants from tumours with one aneuploid and one diploid tumour cell population. This might reflect a more malignant potential of the aneuploid cell clones. This is also demonstrated for SCC of the head and neck, where aneuploid carcinomas had a poorer prognosis than the diploid carcinomas (Holm, 1982).

After a successful primary heterotransplantation there are adaptive changes during the first passages in nude mice which result in a reduction of DT during exponential growth towards a constant limiting value. This varies for different tumour lines (II). Concomitantly there is a shortening of the latency period between inoculation of the tumour specimen and measurable growth (II). This has also been observed in colon adenocarcinoma (Osieka et al, 1977) and epidermal lung tumours (Mattern et al, 1980).

DT reflects by definition only the net growth rate, i.e. the difference between the rate of cell multiplication and cell loss (Shackney et al, 1978). The shortening of DT during the

first passages is parallel with an increase in 3HTdR incorporation into DNA (I). These changes can be explained either by an increased growth fraction (GF), shortened cell cycle time (Tc) or a decreased cell loss or a combination of these factors. Studies of colon adenocarcinomas transplanted to immune-suppressed mice (Pickard et al, 1975; Houghton & Taylor, 1978) showed little change in Tc, cell cycle phase durations and GF during the first passages, while both DT and cell loss factor decreased. There are no similar studies on SCC of the head and neck, but it seems reasonable to assume that also in this type of tumour the changes observed are attributable to a decrease primarily in cell loss factor.

When established SCC tumour lines are compared with SCC of the head and neck in situ in the patient, the DTs of the former (II) are considerably shorter than DTs of the latter, which are reported to range between 13 and >107 days for primary tumours (Bresciani et al, 1974), between 2.8 and 107 for recurrences and between 15 and 320 days for pulmonary metastases (Galante et al, 1982). The Tc of SCC of the head and neck in situ in man, lies in the range of 1.0 to 3.7 days (Frindel et al, 1968; Bresciani et al, 1974; Tannock, 1978). Since in colon adenocarcinomas heterotransplanted to immune--suppressed mice the GF and Tc are not very different from the corresponding tumours in man (Pickard et al, 1975; Houghton & Taylor, 1978; Lamerton & Steel, 1975), it is reasonable to believe that also Tcs of heterotransplanted SCC are close to the Tcs of SCC of the head and neck in situ.

The difference in DT between heterotransplanted tumour lines and SCC in situ is also reflected in differences in the β_2m concentration of tumour tissue (III). β_2m concentration correlates with DT (Michael et al, 1980; III) in that heterotransplants with short DT have a low β_2m concentration and vice versa. When heterotransplanted SCC are compared with patient tumours the mean β_2m concentrations are ten times higher in patient tumours than in heterotransplants. This is in accordance with longer DTs of SCC in situ than as heterotransplants. The correlation between DT and β_2m concentration, makes β_2m concentration a possible parameter in the clinical assessment of tumour growth.

β_2m is associated with the major histocompatibility complex and thus the regulation of cell communication. Low β_2m concentration in tumour tissue might reflect a suppressed β_2m expression on the cell surface. This may lead to the loss of communication with neighbouring cells and a withdrawal of external growth control (III). Determination of β_2m concentration might thus give an indication of the growth rate of the tumour.

CHEMOTHERAPEUTICAL CONSIDERATIONS

Heterotransplanted tumours retain the pattern of drug sensitivity/resistance of the original tumours. In contrast to tumours in man, heterotransplanted tumours allow controlled analysis of the growth pattern before and after manipulation (Sordillo et al, 1980). In the interpretation of chemotherapeutically induced changes it is however necessary to take differences in growth and cell kinetics into consideration.

The growth retardation achieved with cytostatic treatment is dependent on changes on the cellular level. Stragand et al (1982) recently emphasized that the changes in tumour volume increment after cytostatic treatment are correlated to several different events on the cellular levels; cell kill and -renewal, necrosis, oedema, changes in stromal tissues etc. Disturbances on the cellular level are however not necessarily linked to changes in tumour growth. The authors report of a study where a patient with a malignant melanoma was treated with cytostatics. The tumour revealed distinct cell kinetic changes, but did not show any suppression of tumour growth. This information is important when dose-response relationships in vivo are interpreted. In vivo studies based on tumour volume mesasurements should preferentially be combined with cell kinetic studies to yield further information.

MITOMYCIN C

The effect of MMC on the volume growth of heterotransplanted tumours was demonstrated in 1977 (Ovejera et al, 1977) in a colon adenocarcinoma. Hirokawa et al (1981) have reported of a dose-response relationship in the treatment of heterotransplanted head and neck tumours with MMC. They gave iterated doses in the range 0.5 - 2.0 mg MMC/kg b.wt. during four weeks.

On the cellular level MMC induces a block at the S/G2+M transition (IV). This block has also been demonstrated in an in vitro cultivated colon adenocarcinoma line (Barlogie & Drewinko, 1980). It is consistent with the finding that in vitro cultivated cells are most sensitive during late G1 and early S phases (Nowell, 1964; Ørstavik, 1972). Exposure times for MMC are not comparable, however, since the cells were exposed to MMC in vitro for intervals between one and 48 hours, and the biological half life of MMC in man is about 6 minutes (Powis et al, 1981) and is probably also short in mouse. The MMC induced block results in a transient increase of the fraction of cells in S phase. This accumulation is preceded by an increase in 3HTdR incorporation in DNA, which might represent an increased DNA repair after MMC inflicted damage, since it is known that MMC does not inhibit repair mechanisms (Rauth et al, 1970).

CISPLATIN

CDDP has been classified as a cell cycle (phase)-non specific agent, effective against cells in all phases of the cell cycle but most toxic to cells in G1 phase (Meyn et al, 1980; Sigdestad & Grdina, 1981).

Few reports concerning the effect of CDDP on volume growth of heterotransplanted tumours have been published. Yoshida et al (1979) treated a heterotransplanted choriocarcinoma and embryonal carcinoma of the testes with 3 mg CCDP/kg b.wt./day x 5 and Helson (1980) treated a malignant schwannoma with 2 mg CDDP/kg b.wt. in iterated doses and noted retardation of tumour growth.

In heterotransplanted SCC CDDP induces both a G1/S block, with a transient depression of DNA synthesis, and a block or delay of the transition in the G2+M phase (IV). The CDDP induced cell cycle phase perturbations are similar to the changes seen in an in vitro cultivated colon adenocarcinoma line (Bergerat et al, 1979).

BLEOMYCIN

The dose-response curve of heterotransplanted SCC after single injections of BLM show a plateau-phase (V) indicating either resistant clones, different resistance between cycling and non-cycling cells or, since BLM has a short half life in vivo, differences in sensitivity between different cell cycle phases.

BLM has been claimed to block cell progression in the early G2 phase near the S-G2 boundary and within the cell cycle kill mitotic and G2 phase cells most effectively (Barranco, 1978). Furthermore, non-dividing cells seemed to be most sensitive. Other authors, however, were not able to verify this sequence of blocking (Thorud & Clausen, 1980).

FCM analysis after one single i.p. BLM injection revealed only small cell cycle perturbations (V) and were rather in accordance with an accumulation of cells in G1 phase, followed by a synchronized passage of cells through the cell cycle, than a block in the G2 phase. This mode of action on SCC is also supported by findings in epidermal mouse cells by Thorud & Clausen (1980) who demonstrated an inhibition of the cell cycle progress in either the G1 phase or at the G1/S boundary. It might be possible to induce more profound perturbations by using continuous infusion, since it has been shown in Lewis lung carcinoma that continuous BLM infusion enhances the therapeutic ratio (Sikic et al, 1978).

General Summary

Squamous cell carcinoma (SCC) heterotransplanted to nude mice has been evaluated as a model for tumour cell kinetic studies of SCC of the head and neck.

Heterotransplanted tumours allow studies of growth and cell kinetics of SCC under controlled basal and perturbed conditions. Heterotransplanted tumours are not the same as SCC in situ. Heterotransplantation is likely to favour the growth of biologically aggressive tumours and select for aneuploid clones. Growth after direct transplantation of tissue straight from patient tumours is achieved only in about 1/3 of attempted cases and only half of these result in permanent tumour lines, i.e. they can be passed through three or more passages in nude mice. The low frequency of resulting tumour lines and the considerable time needed to establish a tumour line makes the nude mouse system unsuited as a predictive test system for chemosensitivity testing, for example.

The take rate for stage IV tumours seemed to be higher than for less advanced tumours. Take rate is not influenced by the sterility, i.e. biopsy or operative conditions, under which the specimens are obtained. Neither is it ascertained that take rate is influenced by the rate of DNA synthesis or degree of histopathological differentiation.

During the first 3-5 passages there are successive adaptive changes in the growth of the heterotransplants. DT during exponential growth is shortened towards a constant value, different for different tumour lines, the latency period between inoculation and measurable growth is shortened and DNA synthesis increased. The cell cycle time (Tc) and growth fraction (GF) are likely to remain relatively unchanged, while the cell loss factor decreases.

Human β_2m was detectable both in the plasma of tumour-bearing nude mice and immunohistochemically in all the heterotransplanted tumours. The β_2m concentration of the heterotransplanted tumours correlated to their DT. This makes β_2m concentration in SCC of the head and neck a possible indicator of tumour growth. Mean β_2m concentrations were ten times higher for SCC in situ than in heterotransplanted tumour lines, which is in accordance with longer DTs for tumours in situ than for heterotransplants.

Mitomycin C (MMC), cisplatin (CDDP) and bleomycin (BLM) induced dose-dependent inhibition of tumour growth. The shape of the dose-response curve for BLM differed from those of the

others, in that it exhibited a marked plateau-phase. Tumour regression was not observed.

On the cellular level treatment with MMC and CDDP induced reversible blocks at different stages of the transition of cells through the cell cycle, but did not cause any histopathologically detectable cell kill.

BLM induced relatively small cell cycle perturbations, which can be consistent with a reversible, short-lasting G1/S block, followed by a synchronized passage of cells through the cell cycle.

It is of interest to note that the maxima of the induced cell cycle perturbations for the tested drugs, are in the order of 24 to 48 hours. Since the Tc of heterotransplant is likely to be similar to the Tc of SCC *in situ*, this stresses the importance of scheduling in the combination chemotherapy.

By stepwise evaluation of single drugs in the nude mouse system, as exemplified in this work, it is possible to gain knowledge of the course of induced cell cycle perturbations. On the basis of such knowledge, with respect to known differences between heterotransplants and tumours *in situ*, it is then possible to use the model to test different schedules based on cell kinetics and select the most optimal variants for further clinical consideration.

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HETEROTRANSPLANTATION OF HUMAN HEAD AND NECK TUMOURS INTO NUDE MICE

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Abstract. Multimodality therapy of advanced malignant tumours of the head and neck includes surgery, radiotherapy and chemotherapy. However, the cure rate for these tumours is low and guidelines for the selection and timing of therapy are needed. For such guidelines, tumour cell kinetic parameter studies, e.g. cell proliferation, may be a suitable approach. In the present study an in vivo system for tumour cell kinetic studies, the nude mice system, has been evaluated for malignant human head and neck tumours. The overall 'take' rate for heterotransplanted human tumour grafts was 35%. The take rate was not influenced by the sterility state of the specimen. An advanced tumour stage showed a tendency to a higher take rate than less advanced tumour stages. In the tumour cell kinetic study the rate of DNA synthesis, measured by incorporation of radioactively labelled thymidine ([^{3}H]TdR) into DNA, was analysed in human malignant head and neck tumours and in serially heterotransplanted tumours. The rate of DNA synthesis was found to increase during the first serial passages though the histopathological picture remained unchanged. The increased rate of DNA synthesis may be explained by the recruitment of G_0 cells or by stem cell selection. These findings are discussed and may provide a basis for therapeutic guidelines.

In the treatment of stage III and IV head and neck cancers a multimodality therapy is necessary but despite intensive therapy the cure rate is low (Glick & Taylor, 1981).

For optimal therapy the multimodality approach may also require the inclusion of chemotherapy (Ervin et al., 1981) in addition to surgery and radiotherapy. The response to chemotherapy is dependent on various factors, i.e. the proliferative state of the tumour cell population. In a tumour dominated by non-proliferating cells, i.e. G_0 cells, the response to chemotherapy is usually poor. In contrast, chemotherapy can have a good effect when most of the tumour cells are in a proliferative state (Schabel, 1975; Valeriote & van Putten, 1975). Thus, the study of cell proliferation in malignant tumours of the head and neck may be of clinical importance for guidelines to therapy, i.e. dose schedule and administration sequence for chemotherapeutic drugs.

For such studies, in vivo and/or in vitro methods may be used, One recent in vivo method, the nude mouse system, has been used for tumour cell kinetic studies in various human tumours (for review, see Giovanella et al., 1978).

The aim of our work was to study human malignant head and neck tumours heterotransplanted into nude mice and to evaluate the usefulness of this system for cell kinetic studies of these tumours. The tumour cell population was studied before and at different times after heterotransplantation, with respect to cell morphology and to rate of cell proliferation (rate of DNA synthesis).

MATERIALS AND METHODS

Male and female BALB/c nude mice, 5–8 weeks old, were used. Originally the heterozygous mice were supplied by Gl. Bomholtgaard Ltd, Ry, Denmark, and later bred in our laboratory by mating heterozygotes. The colony was kept under clean but not specific pathogen-free conditions. The mice were kept in sterile cages with sterile coarse sawdust bedding. They were fed sterilized ordinary laboratory food and acidified (pH 2.5–2.7) water *ad*

libitum. Antibiotics were not given. The ambient temperature was 25–26°C.

Specimens for the study were obtained from 54 human malignant tumours of the head and neck. In 4 cases preoperative radiotherapy with a dose of 50 Gy, given for 5 weeks, had been administered 4–5 weeks prior to biopsy; all other tumours were untreated. The distribution of histology and localization of the tumours are seen in Tables I and II. Tumour material for heterotransplantation was taken either 'non-sterile' from the peripheral parts of the tumour at diagnostic biopsy (38 specimens), or 'sterile' from the core of the tumour or from regional lymph node metastases under aseptic conditions at surgical excision (16 specimens). The specimens were transported and kept in Parker 199 tissue culture medium (SBL, Stockholm, Sweden) and cut into small pieces measuring 2×2×2 mm before heterotransplantation (grafting).

The grafting was done under aseptic conditions as soon as possible and in no case later than 3 hours after biopsy or excision. The animals were anesthetized with chloralhydrate i.p. (0.5 mg/g b.wt.). Each tumour was transplanted into 3–4 mice. Each mouse received two tumour grafts, each graft inoculated separately subcutaneously on either side of the back.

Tumour pieces were taken from the primary heterograft and subsequent passages for histological examination. They were fixed in Bouin's solution for paraffin-wax embedding and sectioning. Hematoxylin-eosin and Kryberg staining were used.

The rate of DNA synthesis was measured with the technique described by Håkansson & Tropé (1973). The solid tumour was brought into a suspension by pressing through a stainless steel mesh.

The cell suspension was incubated at 37°C for one hour with tritiated thymidine ([^{3}H]TdR; spec. act. 1.9 Ci/mM) at a final concentration of 2 mCi/ml. The cell suspension was then washed with phosphate buffer, the cells were precipitated with cold trichloroacetic acid, to extract nucleosides and nucleotides. The amount of [^{3}H]TdR incorporated into DNA and the total DNA content of each sample was measured. All tests were performed twice.

By relating the incorporation of labelled thymidine, measured as cpm, to the total amount of DNA in the sample, it is possible to compare samples containing different numbers of tumour cells. The amount of incorporated [^{3}H]TdR per unit DNA in the sample was expressed as a logarithmic function (Håkansson & Tropé, 1973). The formula is:

$$a = 100 \log_{10} \frac{\text{cpm} \cdot k}{\text{DNA}}$$

The cpm is the number of counts registered. Counting was done for 5 min in a Beckman Liquid Scintillator 7000. k is a factor needed to correct for i.a. counting efficiency. DNA is the amount of DNA in the sample determined with the method of Bonting & Jones (1957) and expressed in arbitrary units.

The thymidine uptake in a cell sample, as determined by the above expression, gives near-normal distributed variates.

Two perpendicular diameters of the transplanted tumour were measured with graded calipers and the product of the diameters was recorded as the size of the tumour (Bellet et al., 1979). The tumours were measured weekly.

Criteria for acceptance of the heterotransplanted specimen were defined as growth of the transplant to a minimum size of 4×4 mm within 2 months. At autopsy 30–60 days after transplantation the tumour grafts were taken from the inoculation sites. Samples of the tumour grafts were then brought into cell suspension for DNA synthesis determination. The remaining pieces were taken for histological examination and for transplantation to subsequent generations of nude mice (serial passages).

For statistical analysis the correlation between DNA synthesis (a-values) and graft acceptance was done with the Mann-Whitney rank sum test. Analysis of differences in 'take'

rate were done with the χ^2-test with Yates correction, and changes in DNA synthesis during serial transplantation with Wilcoxon's test for paired observations.

RESULTS

Nineteen out of 54 heterotransplanted human head and neck tumours (35%) grew as localized tumours at one or two transplantation sites. The histopathological classification in relation to the results of grafting are shown in Table I.

Table I. *Histopathological classification, number of heterotransplanted tumours studied and results of grafting*

Histopathology	No.	Graft acceptance: Initial success	Graft acceptance: Serial success
Squamous cell carcinoma			
Well differentiated	8	1	1
Moderately differentiated	21	8	3
Poorly differentiated	17	6	4
Other malignant tumours			
Mucoepidermoid carcinoma	2	2	0
Medullary thyroid carcinoma	2	0	0
Anaplastic thyroid carcinoma	1	0	0
Adnexal tumours	1	0	0
Metastatic malignant melanoma	1	1	1
Fibrosarcoma	1	1	1
Total	54	19	10

Four of the transplanted tumours had received preoperative irradiation. One of the four transplanted tumours showed growth in nude mice.

There was no significant difference in take rate between specimens taken as 'non-sterile' biopsies and as 'sterile' excisions at operation, since 6 out of 16 (38%) 'sterile' specimens and 13 out of 38 (34%) 'non-sterile' biopsies were successfully heterotransplanted (Table III). In no case did heterotransplanted 'sterile' tumour specimens cause infection in the host animal. 'Non-sterile' biopsies did in some cases cause local suppurative infection at the implantation site in some mice, but not in others, though the heterotransplants originated from the same biopsy. Local infection at the implantation site was usually not compatible with tumour growth, though in one case we recorded tumour 'take' and growth in a suppurative infection.

Only one out of 8 well differentiated squamous cell carcinomas was accepted. By contrast, 8 out of 21 moderately and 6 out of 17 poorly differentiated squamous cell carcinomas showed growth on nude mice. Statistical analysis (χ^2-test), however, did not demonstrate any significant difference ($p>0.05$) between degree of differentiation and graft acceptance.

Forty-five out of 46 transplanted squamous cell carcinomas were staged according to the UICC classification (UICC, 1978). The 'take' rate was higher for heterotransplants from clinical stage IV tumours than from less advanced stages, as shown in Fig. 1 *a*, though this tendency was not statistically significant ($0.05\ p<0.1$). The tendency was the same for T-stage as studied in 42 out of 46 squamous cell carcinomas. Thus more advanced T-stages, according to the UICC classification, showed an increased take rate, though not statistically significant (Fig. 1 *b*). The difference in numbers of clinically and T-staged cases is due to inadequate T-staging of the primary tumour in 3 cases with distant spread.

Tumour growth was ususally recognized 2–4 weeks after heterotransplantation. The transplants grew as well-circumscribed tumours and did not infiltrate into surrounding tissues. Metastases to lymph nodes or other organs were not observed at autopsy.

In 10 out of the 19 accepted heterotransplants, permanent tumour lines were established, i.e. tumours could be passed through three or more subsequent transplantations to nude mice (Table I and II). The nine tumours that did not establish permanent lines were all lost during the first three passages.

The histopathological picture of the hetero-

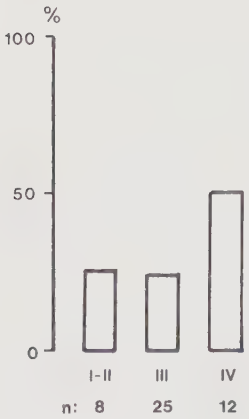

Fig. 1 a. The 'take' rate of 45 squamous cell carcinomas in relation to clinical stage, according to the classification of UICC (1978). *n* denotes number of tumours in each group.

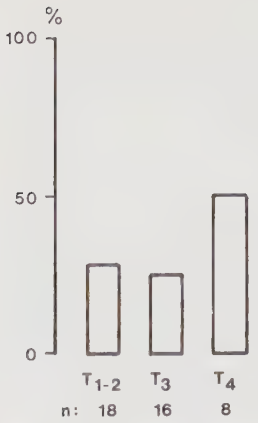

Fig. 1 b. The 'take' rate of 42 squamous cell carcinomas in relation to T-stage, according to the classification of UICC (1978). *n* denotes number of tumours in each group.

transplanted tumours was consistent with the original tumour material. The tumours retained their morphology during serial transplantation.

From 13 tumours the amount of material obtained at surgical excision or biopsy for heterotransplantation was also sufficient to allow determination of the rate of DNA synthesis, measured as incorporation of [^{3}H]TdR and expressed as *a*-values (cf. Methods). The mean *a*-value of those tumours was 107 (left part of Fig. 2).

The rate of DNA synthesis in serially transplanted tumours was followed (Fig. 2). Between the first and second passage in nude mice the rate of [^{3}H]TdR incorporation in tumour DNA was significantly raised ($p<0.05$). When the second and fifth passages were compared, there was no detectable difference in the rate of [^{3}H]TdR incorporation.

Six out of the 13 primary tumours which were analysed with respect to the rate of DNA synthesis, did establish growth when hetero-

Table II. *Localization of heterotransplanted tumours, results of grafting and serial passages*

	No.	Graft acceptance	
		Initial success	Serial success
Oral cavity	20	3	2
Tonsil	9	2	2
Oropharynx	9	6	3
Salivary glands	6	4	1
Skin	1	0	0
Bone	1	1	1
Larynx	4	2	0
Lymph node metastases	4	1	1
Total	54	19	10

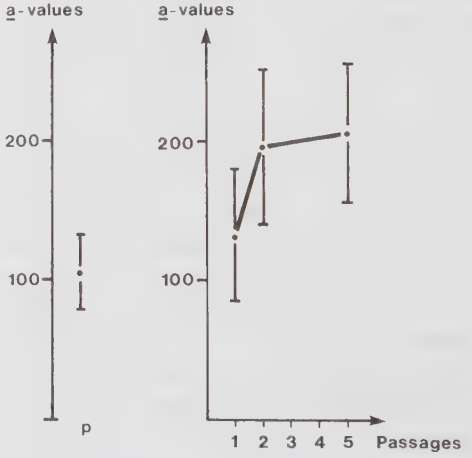

Fig. 2. DNA synthesis expressed in *a*-values ($100 \cdot {}^{10}\log$ [^{3}H]TdR/DNA). *P* denotes primary (= patient) tumour. Numerals denote tumour passage in nude mice. *Points*: mean of 13 tumours (primary tumour) vis-à-vis mean of 6 serially passed tumours in nude mice (passage 1, 2 and 5), *bars*: SD.

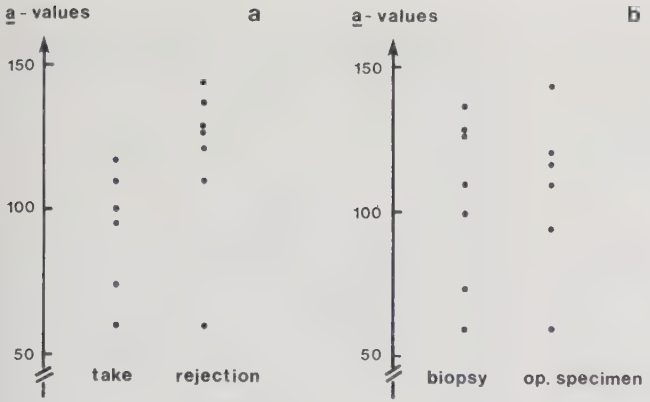

Fig. 3 a, b. Distribution of *a*-values from 13 heterotransplanted tumours, grouped with respect to take vs. rejection (*a*) and biopsy vs. operative specimen (*b*). *Points*: mean of duplicate determinations.

transplanted. There was no statistical difference in *a*-values between tumours establishing vis-à-vis not establishing growth in nude mice (Fig. 3 *a*). Nor was there any difference between specimens taken as 'non-sterile' biopsies and as 'sterile' excisions at operation, as shown in Fig. 3 *b*.

DISCUSSION

The present work was intended to evaluate the nude mouse system as a model for the study of tumour cell kinetics, e.g. cell proliferation, in human head and neck cancers. Most important objects for such studies are advanced squamous cell carcinomas of the head and neck. These tumours constitute a great therapeutic problem and the kinetic studies may provide a better basis for chemotherapy in the multimodality therapy.

The athymic state gives the nude mouse an immunological incompetence that permits acceptance of heterotransplants, e.g. tumour grafts from other species (for review see Giovanella et al., 1978). After acceptance tumour grafts can in some cases be transplanted further in serial passages. During serial transplantation of human heterografts in nude mice the tumours retain different characteristics as cromosome pattern, isoenzyme pattern, antigenicity and histology (Povlsen et al., 1973, 1975). However, no studie on rate of tumour cell proliferation during serial passages seem to have been reported.

Since 1972 when Povlsen & Rygaard reported the transplantation of four human squamous cell carcinomas of the head and neck, few studies on this type of tumour have been reported. The overall acceptance rate of grafts varies. The result in the present study (35%) is consistent with the findings of Lindenberger et al. (1978) who reported growth in 5 out of 14 heterotransplanted tumours (36%) and Fogh et al. (1980) who found growth in 7 out of 28 heterotransplants (25%). Sneeuwloper & Snow (1979) reported growth in 6 out of 35 heterotransplants (17%)

Lindenberger et al. (1978) reported one case of a successful heterotransplantation of a 'non-sterile' biopsy from a laryngeal squamous cell carcinoma. However, no systematic studies on the acceptance of heterotransplanted 'non-sterile' squamous cell carcinomas of the head and neck seem to have been reported. In the present study the heterotransplantation acceptance was the same, irrespective of whether the tumour material was obtained by 'non-sterile' biopsy or by 'sterile' surgical excision (Table III). This information is of importance, since in many cases of squamous cell carcinoma of the head and neck, e.g. in cases of preoperative or full-dose radiotherapy, only 'non-sterile' biopsy material is available prior to therapy.

Table III. *Results of grafting in relation to sterility of specimens*

	No.	Graft acceptance
Sterile specimens	16	6
Non-sterile specimens	38	13

The tendency to higher 'take' rates in the T_4 and stage IV tumours may reflect a more aggressive tumour growth. Our findings are in accordance with the results of Greene (1952) who heterotransplanted human malignant tumours to the anterior chamber of the eye of guinea pigs and found a relationship between the take rate and the subsequent fate of the patient. In his material, patients with transplantable tumours had shorter survival periods than patients with non-transplantable tumours. This difference in biological behaviour is also reflected in the findings of Fogh et al. (1980). In a material of 246 heterotransplanted tumours taken from breast, lung, head and neck, female genitalia, bone tumours and soft-tissue sarcomas, all of varying histological type they found a higher take rate for grafts from metastases and recurrent tumours than from primary tumours without metastases. In the present material the observation time was too short to permit a correlation between take rate and patient survival.

For tumour cell kinetic studies, permanent cell lines are needed. Only 10 out of 54 grafted tumours resulted in permanent cell lines. Similar results have been reported by Fogh et al. (1980). In their material of histologically disparate tumours, permanent cell lines were established in 14%.

With only about one-fifth of heterotransplanted tumours resulting in permanent cell lines the nude mouse system seems to have a limited potential as a tool for prediction of chemotherapy sensitivity. However, the take rate seems to be higher for the most advanced squamous cell carcinomas. Since chemotherapy often is included in the combined treatment of those tumours, the higher take rate may indicate that the nude mouse model in these cases can be used as a valuable predictive test system.

The nude mouse system may also be used in human head and neck cancer for evaluation of new chemotherapeutic agents or to select new combinations of agents. As suggested by Bellet et al. (1979) a panel of grafted tumours of the same histological type, e.g. squamous cell carcinomas of the head and neck, may be suitable for such studies.

In the present study, by using a [^{3}H]TdR-incorporation technique for measuring cell proliferation, the rate of DNA synthesis was found to increase significantly between the first and second serial passages, though the histology was unchanged.

After the rise during the two initial passages in nude mice the rate of DNA synthesis stabilized above the level of the original tumour before heterotransplantation and the first passage in nude mice. These findings are of interest in relation to the results of Povlsen et al. (1973, 1975). They reported not only retained biochemical, histological and immunological identity but also unaltered genetic identity of the tumours during serial passages.

Lindenberger et al. (1978) did not demonstrate any change in DNA distribution in heterotransplanted tumours passed through 1–3 generations of nude mice. Their finding is not inconsistent with an increased DNA synthesis, as found in our material, if this increase is dependent on an increased number of tumour cells passing through a shortened S- and G_2-phase. Recently Fu & Steel (1979), using another technique for measuring cell proliferation, the fraction of labelled mitoses (FLM) method, found that rat mammary carcinoma transplanted into nude mice had a larger fraction of labelled mitoses in the second passage compared with the first. This finding is in agreement with those of the present study.

The increased DNA synthesis may be explained in two different ways; with recruit-

ment of G_0 cells or with stem cell selection. In the first case there may after heterotransplantation be an increased loss of cells. This loss may be an appropriate stimulus for the quiescent G_0 cells to re-enter the cell cycle. In the second case with stem cell selection, the original tumour, viz. the donor tumour, is composed of cell clones with differing capacity to proliferate. During serial passages in a new host there might be a selection of clones of rapidly proliferating cells and/or with a high growth fraction. The ability of a heterotransplant to establish permanent tumour lines might be explained by such changes during adaption to the environment in a new host. Thus heterotransplants lost during the first passages in nude mice might lack this ability to adapt to a new environment.

Should the increased rate of DNA synthesis in the tumour cell population be due to an increased growth fraction, the heterotransplanted tumour may be more sensitive to cell-cycle specific or cell-cycle-phase specific chemotherapeutic agents than the original primary tumour. This shold be considered of importance when the nude mouse system is used to evaluate this type of drug.

ZUSAMMENFASSUNG

Die Behandlung von vorgeschrittenen Stadien der Kopf- und Halstumoren umfasst Chirurgie, ionisierende Strahlung und Cytostatika. Trotz der kombinierten Therapie ist das Behandlungsresultat entmutigend. Um zu einem besseren Resultat zu kommen, braucht man erweiterte Kenntnisse von u. a. tumorzellkinetischen Verhältnissen. In der aktuellen Untersuchung ist ein in vivo System für tumorzellkinetische Studien im Hinblick auf bösartige Kopf- und Halstumoren ausgewertet worden, indem man humane Tumoren auf nackte Mäuse heterotransplantierte. 54 Tumoren wurden heterotransplantiert. Die Anschlagsfrequenz war 35%. Diese Frequenz wurde von der Sterilität des Heterotransplantats nicht beeinflusst. Vorgeschrittene Tumorstadien zeigten eher die Tendenz zur höheren Anschlagsfrequenz als weniger avancierte. In der tumorzellkinetischen Untersuchung wurde die DNA-Synthese mit Inkorporation von radioaktiv gezeichnetem Thymidin gemessen, indem man humane bösartige Kopf- und Halstumoren auf nackte Mäuse serietransplantierte. Es stellte sich heraus, dass die DNA-Synthese während der ersten Passagen bei nackten Mäusen gestiegen war, obwohl das histopathologische Bild unverändert blieb.

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CHANGES IN GROWTH PATTERN OF HUMAN SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK DURING SERIAL PASSAGES IN NUDE MICE

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Abstract

Six human squamous cell carcinomas of the head and neck heterotransplanted to nude mice were studied with respect to changes in tumour volume doubling time (DT) and clonal composition during serial passages. The tumours exhibited a statistically significant decrease in DT between the first and third to fourth passage towards a constant value. The changes in DT are likely to depend on a reduction of cell loss factor. Five of the original tumours and serially passed heterotransplants were analysed with flow cytometry. Two tumours were diploid and remained so after heterotransplantation. Three tumours had one diploid and one aneuploid cell population. At heterotransplantation there was a selection of clones and only the aneuploid cell populations could be recovered from the heterotransplants in the first as well as in later passages.

Introduction

Tumour cell heterogeneity may be defined as different biological properties within a malignant tumour, depending on complex subpopulations of malignant cells (Owens, 1982). The composition and proportions of these subpopulations or cell clones may change during tumour growth, and therefore if these different biological properties include growth characteristics, this might result in a changing pattern of growth compared to the original tumour. Tumour heterogeneity is important for the treatment with chemotherapeutic agents as different cell clones may differ in sensitivity.

Recently, changes in tumour cell kinetics during serial passages of heterotransplanted head and neck carcinomas in the nude mouse model have been studied (Wennerberg et al, 1983 a). The rate of DNA-synthesis increased significantly during the first passages of the tumours. Knowledge of such alterations in growth pattern and tumour cell kinetics in heterotransplanted tumours are of importance for correct interpretation of the effects of chemotherapeutic agents in the nude mouse model. Thus, the aim of the present study was to analyse tumour volume doubling times and clonal composition in squamous cell carcinomas of the head and neck before and after heterotransplantation.

Materials and Methods

TUMOURS: Tumour specimens for transplantation to nude mice and routine histopathological examination were obtained at diagnostic biopsies of squamous cell carcinomas (SCC) of the head and neck. Specimens were kept in Parker 199 tissue culture medium before heterotransplantation. The grafting was done under aseptic conditions and not later than three hours after biopsy. Six successfully established tumour lines, i.e. tumours that could be passed through three or more subsequent transplantations to nude mice, were studied.

Each tumour was transplanted into three or more mice, dependent on the amount of tumour tissue available. Each mouse received two tumour grafts measuring 2x2x2 mm, each graft inoculated separately subcutaneously on either side of the back.

At regrowth the tumours were excised and retransplanted in the same manner.

The method with two separate tumour inoculas on each mouse was used since, in agreement with the findings of Warenius et al (1980) and Spang-Thomsen et al (1980), we did not find any host-induced uniformity in tumour growth of importance.

MICE: For heterotransplantation five to eight week-old male and female BALB/c nude (nu/nu) mice were used. Originally heterozygous mice were supplied by Gl.Bomholtgaard Ltd, Ry, Denmark, and later bred in our laboratory by mating heterozygous (nu/+) females with homozygous (nu/nu) males. The colony was kept under sterile but not specific pathogen-free conditions. The mice were held in sterile cages under filter tops and with coarse sawdust bedding. They were fed sterilized ordinary laboratory food and acidified (pH 2.5-2.7) water ad libitum. Antibiotics were not given. The ambient temperature was 25-26^{o}C.

TUMOUR MEASUREMENTS: Tumour volume was calculated from diameter measurements according to the formula: Volume = length x width^{2}/2, as previously described (Wennerberg et al, 1983 b). The tumours were measured twice weekly.

For each individual tumour the logarithmic value of the calculated volumes were plotted against time. The tumour volume doubling time (DT) was determined from the early exponential phase of tumour growth before elongation of DT

occurred. For each tumour line the median DT of each passage was determined. In each passage in nude mice the take rate was also calculated.

FLOW CYTOMETRIC (FCM) ANALYSIS: Five of the primary biopsy specimen as well as the transplanted tumour lines were analysed. One of the original six tumours, and its corresponding tumour line could not be analysed because of technical error. Until FCM analysis the tumour pieces were frozen at $-70^{\circ}C$ in dimethylsulfoxide/citrate buffer. The procedure for isolation of nuclei and quantitation of DNA in individual tumour cell nuclei has been described in detail by Wennerberg et al (1983 b). Ten thousand to 70.000 propidium-iodide stained tumour cell nuclei were measured in each individual sample in a Cytofluorograf System 50-H (Ortho Instruments, USA).

Human lymphocytes prepared in the same way were used as control to determine the ploidy (DNA-index) of the tumour cell populations. The DNA content of mouse lymphocytes was reported to be 82% of the DNA content of human lymphocytes (Handbook of Biochemistry and Molecular Biology, 1976). Thus it is possible to separate normal mouse cells from normal human diploid cells.

Samples for cytopathological examination were also taken from the cell suspensions prepared for FCM. In each sample 300-500 cells were examined.

STATISTICAL ANALYSIS: Changes in DT were evaluated with Wilcoxon signed rank-test for matched pairs. Changes in lag-phase of the growth curves of a tumour line (TU) between the first and fourth passage were analysed with Mann-Whitney test.

Table I. *DT-time and take rate in relation to passage in nude mice, ploidy and histopathological differentiation*

Tumour	Passage no 1 DT	Passage no 1 Take rate	Passage no 2 Take rate	Passage no 3 Take rate	Passage no 4 Take rate	Primary biopsy Ploidy	Primary biopsy DNA-index	Primary biopsy Histopath. differentiation
		%	%	%	%			
EH	5,6	1/14 (7)	1/10 (10)	10/16 (63)	8/8 (100)	diploid	1,0	poorly
HH	21,5	3/12 (25)	8/18 (44)	4/9 (44)	9/12 (75)	diploid	1,0	high
MW	10,2	4/16 (25)	7/12 (58)	9/15 (60)	8/10 (80)	-		moderately
BH	9,2	6/12 (50)	4/10 (40)	6/10 (60)	1/2 (50)	diploid + aneuploid	1,0 + 1,27	moderately
TU	6,3	7/8 (88)	7/10 (70)	7/8 (88)	5/7 (71)	diploid+ aneuploid	1,0 + 1,59	moderately
JH	5,5	2/4 (50)	3/4 (75)	6/9 (76)		diploid + aneuploid	1,0 + 1,55	poorly

Results

Six tumour lines were studied with respect to tumour volume doubling time. The number of attempted transplantations and resulting tumours in each passage are given in Table I. The tumour lines showed a decrease in median DT between the first and third to fourth passages (Fig. 1). The differences in median DT between the tumour lines were more pronounced in the first passage than in later passages. A statistically significant decrease ($p<0.025$) was found when the median DTs of the first and third to fourth passages of the group consisting of the six tumour lines were analysed. The DTs of the tumour lines showed a tendency to stabilize at a minimum value, different for each line. The DTs of the tumour lines at the fourth passage significantly differed from each other ($p<0.025$).

Poorly differentiated tumours seemed to have a shorter median DT in the first passage than moderately and highly differentiated carcinomas (Table I). Three of the tumours had an initial take rate of <50% (Table I), and in these cases the decrease in DT with subsequent passages was accompanied by an increase in take rate.

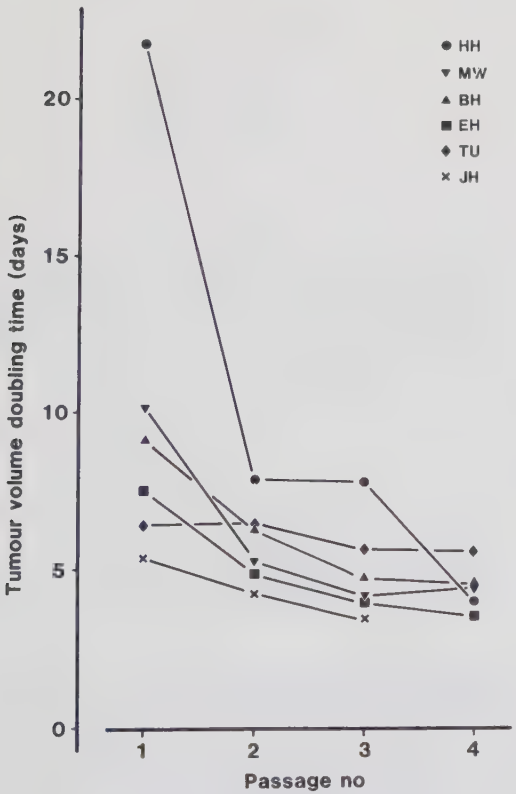

Fig. 1. Curves showing DT of six serially passaged SCC of the head and neck. For each tumour line the median DT-value of each passage is given.

Some tumours were measurable at a diameter of 2 mm, which corresponds to a calculated volume of 4 mm^3 ($=10^{0.6}$ mm^3), and the tumours were always measurable at a diameter of 5 mm, corresponding to a calculated volume of 62.5 mm^3 ($=10^{1.8}$ mm^3). The time between s.c. transplantation and measurable size was reduced between the first and fourth passage as exemplified by the tumour line (TU) in Fig. 2, where the growth curves of the individual tumours of the first and fourth passage are shown. The time needed to reach measurable size is significantly ($p<0.01$) decreased between the first and fourth passage, indicating a decrease of the lag-phase. This is seen despite this tumour had a short DT even from the first passage (Fig. 1).

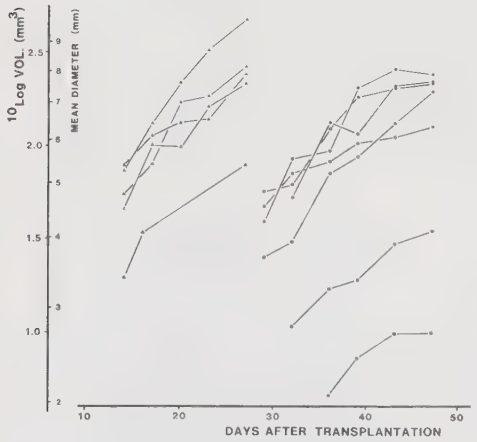

Fig. 2. The growth curves for the individual tumours of the TU tumour line in the first (●) and fourth (▲) passage in nude mice is shown. The time after transplantation is given on the X-axis and the tumour size as ^{10}log volume (mm^3) and corresponding mean diameters are given on the Y-axis.

Two of the primary transplants had a diploid tumour cell population, while the other three had two tumour cell populations, one diploid and one aneuploid (Table I). Examination of cytologic preparations from the latter three tumours showed that the percentage of diploid cells considerably exceeded the percentage of cytomorphologically benign cells (Table II). This suggests that benign cells are not the only component of the diploid peak, and that diploid malignant cells were included.

At heterotransplantation the diploid tumours remained diploid during subsequent passages on nude mice. However, when the diploid + aneuploid tumours were heterotransplanted, only the aneuploid tumour cell population could be detected in the growing heterotransplants in the first and subsequent passages (Fig. 3 a,b).

In the two heterotransplanted tumours with only diploid cell populations it was possible to separate the peak of the mouse stromal cells from the diploid G0+G1 peak of the tumour human cell population. The DNA content of mouse stromal cells was estimated to be 87% of the DNA content of the human tumour cells.

Table II. *Percentage of cytologically benign cells in relation to the percentage of diploid cells in the primary biopsies of three tumours with aneuploid cell populations*

Tumour line	TU	JH	BH
Cytologically benign cells	<1.5%	<0.6%	<5.0%
Fraction of diploid cells	4.8%	18.5%	31.9%

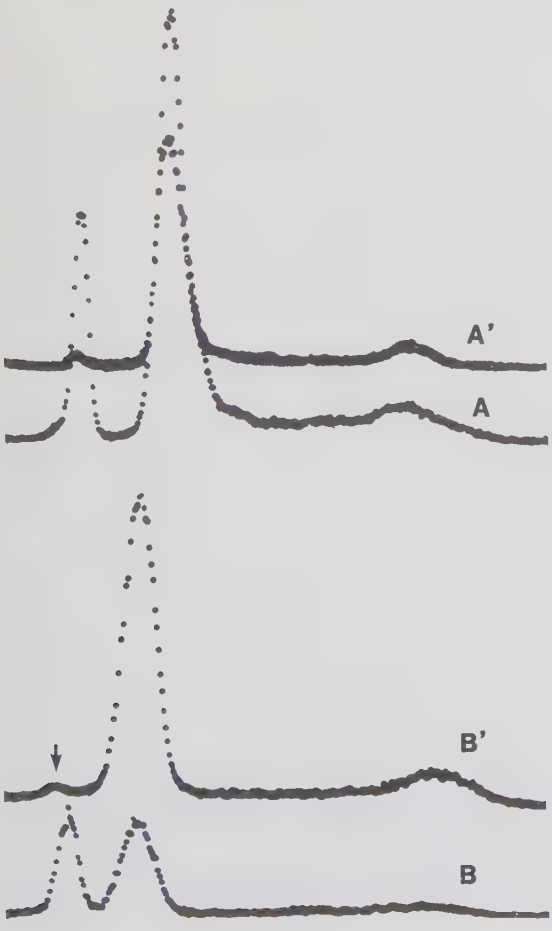

Fig. 3 a,b. The actual DNA histograms of biopsies from two different tumours (A=JH and B=BH) and the resulting heterotransplants (A' and B') are shown. The tumour biopsies have two distinct tumour cell populations, while only the aneuploid populations are represented in the corresponding heterotransplant. The small peak at the left in the DNA histogram from the growing heterotransplant, marked with an arrow in Fig 3 b, represents mouse stromal cells. The maximum of this peak is separated from the maximum of the peak of the diploid cells in the tumour biopsy.

Discussion

This study has demonstrated alterations in growth pattern in head and neck squamous cell carcinomas heterotransplanted to nude mice. During the first passages in nude mice tumour volume doubling time and initial lag-phase were found to decrease. Growth pattern of tumours, transplanted to nude mice, may vary due to tumour type. Our results are supported by the findings of Lamerton & Steel (1975) and Houghton & Taylor (1978) in human colorectal tumours serially passaged in immune--deprived mice, and Mattern et al (1980) in heterotransplanted epidermal lung tumours. In contrast Fodstad et al (1980) in a study of malignant melanoma did not find the changes reported in the present study, which indicates that intrinsic growth potential may be related to tumour type.

DT reflects only the net growth rate, that is, the difference between the rate of cell production and the rate of cell loss (Steel, 1968). A decrease in DT during the first passages in nude mice, as found in the present study, can be attributed to an increase in growth fraction (GF), shortened cell cycle time (Tc) or decrease in cell loss factor. Using the technique of labelled mitoses (PLM) Pickard et al (1975) studied kinetic parameters as cell cycle phase durations, Tc and GF in a heterotransplanted colon adenocarcinoma during its three first passages in immune-suppressed mice, but found little change in the proliferative state of the tumour cells. Houghton & Taylor (1978) also studied cell-proliferative kinetics of a colon adenocarcinoma in its third and tenth passage in immune--suppressed mice. The DT decreased from 11.1 to 6.6 days, with little concomitant change in Tc and GF, but a decrease in cell loss factor from 86% to 76%. The authors attributed the decrease in DT mainly to the change in cell loss. It is thus reasonable to assume that the heterotransplanted tumours in the present study may have a relatively constant cell cycle time (Tc) and the changes in DT during the first passages in nude mice would thus be attributed to a decrease in cell loss.

The DT of the established tumour lines in the present study varies between three and five days. This is considerably lower values than has been reported of from SCC of the head and neck in man in vivo by Bresciani et al (1974). They found DTs ranging between 12 and >107 days. Since the growth fraction even in rapidly growing experimental animal tumours is less than 100% (Steel, 1977), it is reasonable to assume that the Tc of the established tumour lines in this study is less than their tumour volume doubling times, that is, less than 3-5 days. This is in the range of mean Tcs between 1.0 and 3.7 days reported of from SCC of the head and neck in man (Frindel

et al, 1968; Bresciani et al, 1974; Tannock, 1978). The difference in DT of SCC of the head and neck in man and in heterotransplants might thus depend on changes in cell loss. This is supported by studies of a rat mammary tumour transplanted into immune-suppressed mice (Fu & Steel, 1979), changes in heterotransplanted large bowel tumours (Lamerton & Steel, 1975) and in heterotransplanted malignant melanoma by Rofstad et al (1982).

The concept of tumour heterogeneity is well established (Håkansson & Tropé, 1974; Fidler & Kripke 1977) and the occurrence of cells with different karyotypes within a tumour has been demonstrated both in animal and human tumours. The present study demonstrates a loss of the diploid cell population when diploid + aneuploid tumours are heterotransplanted. If the diploid cell population is composed entirely of benign cells this could explain the absence of diploid cells in the heterotransplanted tumour. However, the percentage of cytologically benign cells is considerably lower than the percentage of diploid cells in the tumours, which indicates a selection of aneuploid tumour clones at heterotransplantation. It is known that in SCC of the head and neck, diploid carcinomas are mostly small tumours and aneuploid tumours show tendency toward more advanced clinical stages (Holm et al, 1980). One explanation could be that the diploid carcinomas might be less malignant with slower progression than non-diploid tumours. On the other hand there might be an increase of the degree of aneuploidy as the carcinomas progress. Transplantation of human tumours into nude mice may act in a similar way, and thus promote a selection of one of the clones in the originally heterotransplanted human tumour, as demonstrated in the present study. A similar event has been reported by Vindelöv et al (1982), who in a heterotransplanted human colonic carcinoma line noted the appearance and overgrowth of a population of cells with a DNA content twice that of the original population, which completely disappeared. There might also be an association between biological aggressiveness and selection of aneuploid clones at heterotransplantation since it is known that aneuploid carcinomas of the head and neck have a worse prognosis than diploid tumours (Holm, 1982).

The finding of clonal selection at transplantation of human SCC to nude mice is of interest, since it has been shown that clones within animal tumours (Håkansson & Tropé, 1974), human tumours (Tropé et al, 1975; Tropé et al, 1979; Biörklund et al, 1980) and *in vitro* cultivated tumour cell lines (Dexter et al, 1978; Chu et al, 1979; Dexter et al, 1981) can differ not only with respect to karyotype, but also in morphology, growth kinetics and sensitivity to chemotherapeutic agents.

The present study demonstrates important differences between heterotransplanted tumours and their human counterparts both with respect to growth characteristics and clonal composition. The findings are of importance, since the nude mouse system is used for experimental chemotherapy of human tumours (c.f. Giovanella, 1980) and the response to cytostatic agents is

influenced by the growth kinetics of the treated tumour (Schabel, 1975; Valeriote & van Putten, 1975; Shackney et al 1978). In order to properly interpret the drug induced changes in heterotransplanted tumours exposed to chemotherapy it is thus important not only to stress similarities between human and heterotransplanted tumours, but also to define the differences in cell and tumour growth kinetics.

Acknowledgements

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β_2-MICROGLOBULIN IN SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK IN TUMOURS HETEROTRANSPLANTED INTO NUDE ATHYMIC MICE

by

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III

Abstract

β_2-microglobulin (β_2m) is a small polypeptide, related to the immunoglobulins and present on nucleated cells as part of the strong transplantation antigens. Raised plasma levels have been recorded i.a. in patients with various malignant diseases. Human β_2m has also been immunohistochemically detected in carcinomas transplanted to nude mice, and in the plasma of tumour-bearing mice. In the present study the occurrence of human β_2m was studied in the plasma of 26 patients with squamous cell carcinoma (SCC) of the head and neck and in six heterotransplanted carcinoma lines. Extractable β_2m was measured in seven SCC of the head and neck and in the six heterotransplanted tumour lines. Immunohistochemically detectable β_2m was studied in 20 SCC and in six heterotransplanted tumour lines. Only three of 26 patients (12%) had raised p-β_2m. Stage IV tumours seemed to have higher p-β_2m (though within the normal range) than did less advanced tumours. There was no correlation between the total amount of extractable β_2m in the patient tumours and p-β_2m. However, there was an association between the concentration of β_2m in the tumours and p-β_2m. Human β_2m could be detected in the plasma of mice with tumours from all tumour lines, and there was a tendency toward an association between tumour size and p-β_2m. The ratio of p-β_2m/tumour volume differed between the tumour lines. Tumour volume doubling time (DT) was determined for the different tumour lines, and a correlation was possible between DT and β_2m concentration of the tumours. The mean β_2m concentration was ten times higher in patient tumours than in heterotransplanted tumours, which indicates faster growth of heterotransplanted tumours than of tumours in man. Membrane bound β_2m was immunohistochemically detectable both in SCC of the head and neck and in heterotransplanted tumours. The implications of the findings of the present study for the use of β_2m as a tumour marker and the importance of the association between concentration of extractable tumour β_2m and DT are discussed.

Introduction

β_2-microglobulin (β_2m) is a small polypeptide (molecular weight 11.800), which is structurally related to immunoglobulins and localized at the surface of nucleated cells as part of the strong transplantation antigens (human lymphocyte antigen) (c.f. Berggård et al, 1980). In normal human tissue it is also found in various biological fluids (Berggård & Bearn, 1968). Cells derived from the extraembryonic mesenchyme (endothelial, lymphoid and macrophage cells) are particularly rich in β_2m, as described in an immunohistochemical study by Governa & Biguzzi (1976).

The plasma concentration of β_2m (p-β_2m) rises in patients with renal failure (Karlsson et al, 1980), immunologically related diseases and various malignancies such as bronchogenic carcinomas (Shuster et al, 1976), lymphoproliferative disorders (Cassuto et al, 1978), breast and gastrointestinal carcinomas (Teasdale et al, 1977) and gynecologic carcinomas (Tropé et al, 1980). In head and neck carcinomas significantly raised plasma levels have also been found, but only in 13 of 152 cases (8.5%) (Viot et al, 1978). In a preliminary report (Biörklund et al, 1980) human β_2m was detected in the plasma of nude mice heterotransplanted with human squamous cell carcinomas of the head and neck.

β_2m has been discussed as a possible tumour marker in, among others, lymphoproliferative disorders (Cassuto et al, 1978) and gynecologic tumours (Tropé et al, 1980). Thus it was considered of interest to further investigate the presence of β_2m in human squamous cell carcinomas of the head and neck, including tumours transplanted to nude mice, and to analyse the relationship between p-β_2m, β_2m extractable from tumour tissue and growth rate of tumours in patients with squamous cell carcinomas and nude mice heterotransplanted with those tumours.

Materials and Methods

Human tumours

At diagnostic biopsies blood was sampled for p-β_2m and s-kreatinine determination from 27 patients with previously untreated squamous cell carcinoma (SCC) (22 with oral or pharyngeal, 1 with a maxillary and 4 with laryngeal carcinomas). In 20 cases tumour specimens were taken for light microscopical histopathological evaluation and immunofluorescence examination. In 16 of these patients specimens were also taken for transplantation into nude athymic mice. From the other 7 patients tumour specimens were taken for determination of extractable tissue-β_2m.

The tumours were staged according to the UICC classifications (1978). The volumes of the primary tumours were estimated according to the formula: length x width x height/2 (Steel, 1977).

Heterotransplanted tumours

In 16 patients tumour specimens were obtained for heterotransplantation. Tumour specimens, measuring 2x2x2 mm, were implanted s.c. in the back of 3 to 5 nude mice. Each mouse received 2 heterotransplants, one on each side. A tumour line was regarded as established when the heterotransplanted tumour had been passed through three or more generations of nude mice. The tumour was then transplanted to between 5 and 10 mice. Two perpendicular diameters of the tumours were measured twice weekly with graduated calipers. Tumour bearing animals were sacrificed when the tumours had reached diameters between 4 and 30 mm. Tumours were excised and weighed. Tumour volume was calculated according to the formula: length x width^2/2 (Steel, 1977). Tumour volume doubling time (DT) i.e. the time needed to double the volume of the tumour, was determined from the phase of exponential growth.

Concentration of tumour-β_2m was determined in six tumour lines. Five of these lines originated from the 16 attempted heterotransplantations in this study; one was a carcinoma line established previously in our laboratory.

Plasma sample collection

Blood samples from patients were obtained by cubital vein puncture and collected in heparinized syringes. After centrifugation at 800 g for 10 minutes, the supernatant plasma was stored at -20°C until analysis. Blood from nude mice was obtained through cardiac puncture and then treated as described above.

Extraction of β_2m from solid tumour

The tumour specimen was weighed and then minced with scissors in 0.9% NaCl. The suspension was homogenized in an all glass Potter-Elvehjem homogenizer using an ice bath to avoid elevation of temperature. The homogenate was stored at -70°C until analysis. It was then thawed and centrifuged at 105.000 g for one hour. The supernatant was collected and diluted. β_2m concentration (µg β_2m/mg tumour tissue) was determined as described below. In order to estimate the total amount of extractable β_2m (tumour-β_2m) from the calculated tumour volumes, the density of the tumours was assumed to be 1 g/cm^3.

Radioimmunoassay for human β_2m

Human β_2m was purified from urine by a modification (Berggård et al, 1980) of the original procedure (Berggård & Bearn, 1968). The protein was radiolabelled by the Chloramin T method (Greenwood et al, 1963). It was then used in a radioimmunoassay described by Plesner et al (1975) together with a goat antihuman β_2m antiserum, produced according to Björck et al (1977). Immune-complexes were separated from unbound ^{125}I-human β_2m by polyethylenglycol exclusion (PEG 6000, Kebo AB, Stockholm, Sweden). The β_2m concentrations were determined at final dilutions of 1:250 (human plasma) or 1:25 (mouse plasma). At such dilutions, the β_2m of mouse plasma did not interfere with the assay. The interassay variation, estimated by multiple determinations on selected plasma samples, was approximately 10%. The upper limit of normality (mean + 2 S.D.) in man was defined from control material previously reported (Tropé et al, 1980).

Nude mice

BALB/c nu/nu mice were used for heterotransplantation. They were bred in our laboratory. The colony was kept under sterile but not specific pathogen-free conditions (Wennerberg, 1983).

Light microscopy

For histopathological examination tissue specimens were fixed in 10% formol, dehydrated and embedded in paraffin. Sections were cut at a thickness of 6 µ and stained in haematoxylin and eosin.

Immunofluorescence technique

Tissue specimens were frozen in isopentane quenched to the temperature of liquid nitrogen, in which they were then collected and further stored. For further processing by the indirect immunofluorescence method of Coons (1958) cryostat sections were cut at a thickness of 8 μ, melted to glass slides and air dried. They were incubated for 30 minutes at room temperature in rabbit-antihuman β_2m diluted in phosphate buffered saline (PBS) with 5% normal swine serum (NSS) to titres of 1/20, 1/40, 1/80, 1/160 and 1/320. After washing in PBS for 5 minutes the slides were incubated for 30 minutes at room temperature in fluorescein isothiocyanate (FITC)--conjugated swine anti-rabbit immunoglobulin diluted to 1/20 in PBS with 5% NSS. After washing in PBS for 5 minutes the slides were mounted in PBS with 10% glycerol. Controls were performed according to Coons (1958) and Malmnäs Tjernlund & Forsum (1977). (All antisera and normal swine serum were purchased from DAKO Immunoglobulins AB, Stockholm, Sweden.)

Statistical analysis

Evaluation of the correlation between significantly raised p-β_2m levels and clinical tumour stage was done with χ^2-test. Curve analysis was carried out with linear regression. All other statistical calculations were done with analysis of variance (ANOVA).

Results

STUDIES IN PATIENTS

Tumour extension and p-β_2m

One of the patients was excluded because of raised s-kreatinine. Since impaired renal function can raise the level of p-β_2m (Karlsson et al, 1980), it was possible to evaluate only 26 patients with respect to p-β_2m in relation to histopathological evaluation, TNM-classification and clinical stage.

For the 26 cases there was no correlation between p-β_2m and degree of tumour differentiation (p=0.360). The distribution is given in Table I. There seemed, however, to be an association between clinical stage and p-β_2m. Stage IV tumours exhibited higher p-β_2m values than stage III tumours (p=0.019, Table II). The presence or absence of regional and/or distant metastases did not seem to influence the p-β_2m level (p=0.205, Table III).

Table I. *Relation between degree of histopathological differentiation of squamous cell carcinomas of the head and neck and p-β_2m*

Histopathology	p-β_2m (mg/l) Mean ± SD	n
Anaplastic - poorly differentiated	1.42 ± 0.38	11
Moderately differentiated	1.54 ± 0.76	9
Well differentiated	1.13 ± 0.31	6
Total	1.39 ± 0.53	26

Table II. *Relationship between clinical stage of squamous cell carcinomas of the head and neck and p-β_2m*

Stage	p-β_2m (mg/l) Mean ± SD	n
III	1.26 ± 0.32	17
IV	1.80 ± 0.71	6
Total	1.40 ± 0.50	23

Table III. *Relationship between metastasis of squamous cell carcinomas of the head and neck and p-β_2m*

TNM	p-β_2m (mg/l) Mean ± SD	n
T1-4 N 0 M 0	1.52 ± 0.65	14
T1-4 N ≥ 1 M ≥ 1	1.25 ± 0.31	12
Total	1.39 ± 0.53	26

When related to the normal p-β_2m distribution (Tropé et al, 1980), only three of the 26 patients (12%) had p-β_2m raised above the normal level (>2.0 µg/l). In two of the three patients the tumours were moderately differentiated and, in the third patient, poorly differentiated. Tumour extension in two of these patients corresponded to clinical stage II and, in the other patient, to clinical stage IV.

Tumour-β_2m and p-β_2m levels

β_2m was extracted from 7 oral and pharyngeal tumours in patients with p-β_2m within normal levels. The amount of extractable tumour-β_2m ranged between 0.58-4.50 µg/mg tumour. The calculated sizes of the tumours varied between 1 and 35 cm^3 and the estimated total amount of β_2m in each tumour ranged between 2.3 and 81.0 mg.

There was no correlation between p-β_2m and the calculated total amount of β_2m in the tumour ($r=-0.0848$; $p>0.20$). Neither was there any statistically significant correlation between extractable β_2m/mg tumour tissue and p-β_2m ($r=0.668$; $0.05<p<0.10$) (Fig. 1).

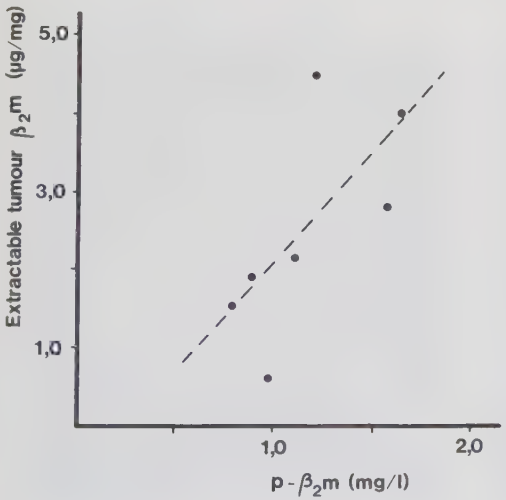


Fig. 1. Extractable β_2m/mg tumour tissue in squamous cell carcinomas in man vs. p-β_2m ($r=0.6678$; $0.05<p<0.10$).

STUDIES IN NUDE MICE

Heterotransplanted tumours

Five of 16 heterotransplanted tumours (31%) resulted in established tumour lines. All of them produced human β_2m, which could be detected in plasma of tumour-bearing mice. Only one of the tumour lines originated from a patient with elevated p-β_2m.

When these lines, together with a previously established tumour line, were studied, there was an association between p-β_2m and tumour weight for each individual line (r ranging between 0.220 and 0.698; $p>0.10$ in all cases). The distribution is illustrated in Fig. 2. This tumour (r=0.269) was one of the fast growing tumours.

Determination of tumour volume doubling time (DT) and extractable β_2m from heterotransplanted tumours was carried out on six tumour lines. The DTs varied between 2.95 and 6.46 days. Extractable human β_2m varied between 0.075 and 0.66 µg/mg tumour. The amount of extractable β_2m exhibited a correlation with DT (r=0.8734; $0.02<p<0.05$) (Fig. 3), showing that fast growing tumours have less extractable β_2m than slow growing tumours.

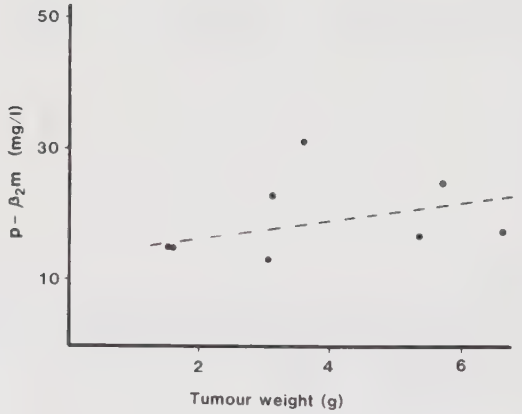


Fig. 2. The relation between p-β_2m and tumour weight in a fast growing heterotransplanted tumour line established from a poorly differentiated squamous cell carcinoma of the nose (r=0.2691; $p>0.10$).

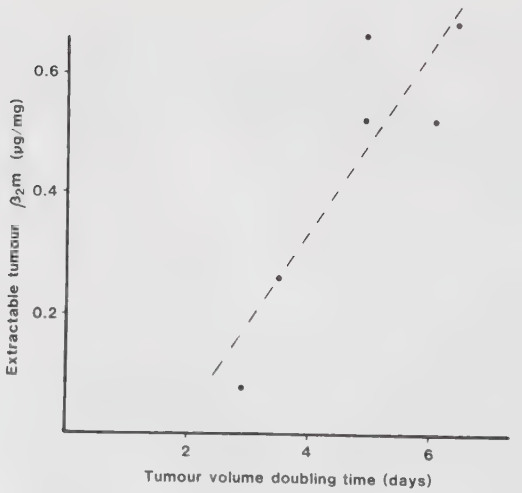
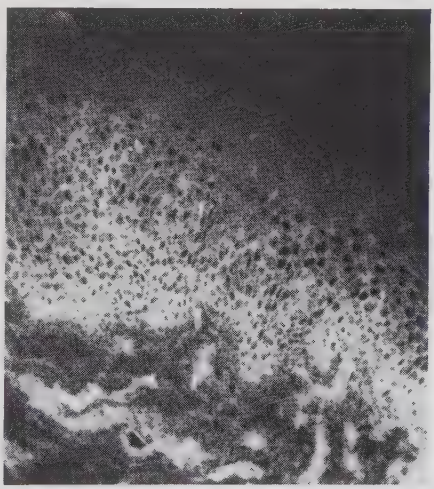


Fig. 3. The relation between extractable β_2m/mg tumour tissue and tumour volume doubling time for heterotransplanted tumour lines ($0.02<p<0.05$).

β_2m immunofluorescence pattern in normal and tumour tissue

In normal squamous epithelium from all parts of the oral cavity, oro-, naso- and hypopharynx, the immunofluorescence pattern was the same. There was a specific immunofluorescence which was smooth, gracile and linear and had a membrane-like pattern around the cells in approximately the basal and half of the epithelial layers, whereas in the corresponding superficial half of the epithelial layers it was absent (Fig. 4 A). In the subepithelial connective tissue no specific β_2m immunoreactivity was found.

All tumour specimens (both from biopsies of the patients and from tumours growing in nude mice) displayed specific tumour cell immunofluorescence which also had a membrane-like pattern but had a more varying appearance compared to normal squamous epithelium (Fig. 4 B), from linear and smooth to granular and irregular. Further, in more differentiated tumour cells the membrane pattern was seemingly more coarse and had a more intense fluorescence intensity (when comparing fluorescence intensities after incubation with various titres of β_2m antisera, and in comparison with the fluorescence intensities of the positive controls). In areas with prominent keratotic differentiation no specific immunofluorescence could be demonstrated.



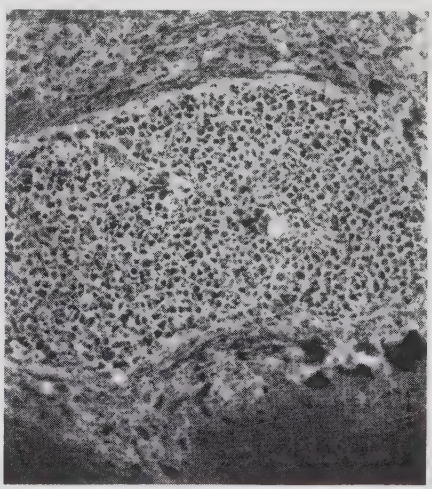


Fig. 4A. Oral squamous epithelium with ordinary differentiation and outgrowth. In the basal half of the epithelial layers, the cells are surrounded by specific β_2m immunofluorescence with a smooth, linear and membrane-like character. Fluorescence micrograph, x260.

Fig. 4B. Specimen from a moderately differentiated squamous cell carcinoma of the lingual tonsil. All cells are surrounded by a membrane-like, specific β_2m immunofluorescence, which is irregular and sometimes granular. Fluorescence micrograph, x200.

Discussion

Though advanced disease seemed to be associated with elevated p-β_2m levels within normal levels, there was no correlation between calculated total amount of β_2m in the tumours and p-β_2m. In the patient material there was a probable association between the concentration of β_2m in the tumours and p-β_2m. It is thus not the total amount of tumour β_2m that seems to be the major determinant of p-β_2m, but rather the ability of tumour cells to release β_2m into circulation. Furthermore, the present data show that, although β_2m was both extractable from human SCC and immunohistochemically detectable, only 12% of the patients had elevated plasma levels, which confirms the opinion of Viot et al (1978) that β_2m is not likely to be of value as a tumour marker in detection of SCC of the head and neck.

The finding of a relationship between tumour size and p-β_2m for the individual, heterotransplanted tumour lines, however, indicates the possibility of using p-β_2m as a complement to physical examination of the patient in monitoring tumour response to therapy and of detecting recurrent disease through consecutive measurement of p-β_2m during and after treatment.

The amount of extractable β_2m/mg tumour shows a ten-fold range of difference both in heterotransplanted tumours and in patient tumours. However, the concentrations of β_2m (μg β_2m/mg tumour tissue) are ten times higher in the latter category. Human tumour biopsies for β_2m determination were to some extent contaminated with blood, in contrast to heterotransplanted tumours, which were taken from exsanguinated animals. Plasma contamination cannot, however, explain the difference in tumour-β_2m, since the mean β_2m concentration in human tumours was more than one thousand times higher than in plasma.

The finding of a positive correlation between DT and concentration of β_2m in the heterotransplanted tumours is in accordance with the results of Michael et al (1980), who studied nude mice with tumours established from *in vitro* cultivated human tumour lines. Differences in extractable β_2m between tumours might thus reflect differences in growth rate. This can explain the observed difference in tumour-β_2m concentration between heterotransplants and tumours in man, which thus may reflect differences in tumour DT. This conclusion is supported by previous findings (Wennerberg, 1983), where DTs of heterotransplanted SCC of the head and neck were shorter than DTs of SCC of the head and neck in man. The ten-fold range of difference in β_2m concentration in human tumours might thus also be explained by differences in growth rate.

The immunohistochemically detectable β_2m was evenly distributed both in primary tumours and in heterotransplanted tumours. This is in contrast to the findings of Weiss et al (1981), who found heterogenic distribution patterns of immunoreactive β_2m in infiltrating ductal carcinomas of the breast but not in benign human breast tissue. The finding can indicate a more homogeneous clonal composition of SCC of the head and neck than of carcinomas of the breast.

β_2m is associated with major histocompatibility complex (MHC) antigens and thus connected to the regulation of cell communication. Dr Abraham Shalev (Ben Gurion University of the Negev, Israel, pers. comm.) has proposed a model where the β_2m gene is suppressed or permanently inactivated during tumour progression. This may lead to the loss of communication with neighbouring cells and to increased growth rate, to escape from recognition by immune systems, and to metastatic spread (c.f. Robbins & Fudenberg, 1983). The model is supported by the finding of a relationship between tumour volume doubling time and extractable tumour β_2m in the present study. Determination of the concentration of β_2m in squamous cell carcinomas of the head and neck might thus give an indication of growth rate and biological aggressiveness of the tumours. To ascertain this, however, prospective studies are needed.

Acknowledgements

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CELL CYCLE PERTURBATIONS IN HETEROTRANSPLANTED SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK AFTER Mitomycin C AND Cisplatin TREATMENT

by

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Abstract

Cell kinetic studies are of interest for clarifying the concepts of chemotherapeutic strategy in the multimodality therapy of advanced squamous cell carcinoma of the head and neck. A poorly differentiated squamous cell carcinoma of the head and neck heterotransplanted to nude mice was used for analyses of chemotherapeutically induced cell cycle perturbations. The heterotransplanted tumour, in its 15th or later passage on nude mice, was treated either with mitomycin C or cisplatin. After determination of dose-response relationships and toxicity, treated tumours were biopsied at different intervals and cell cycle distribution pattern, 3HTdR incorporation into DNA, histology and tumour volume were recorded. Mitomycin C and cisplatin gave the same pattern of cell cycle perturbations, although the changes induced by cisplatin were more profound. There was an initial, transient increase of the fraction of cells in the S phase, concomitant with a reduction of the fraction of cells in G0+G1 phase. When these perturbations were normalized a transient increase of the fraction of cells in G2+M phase was observed. However, while cisplatin caused an initial transient depression of DNA synthesis, the mitomycin C treated tumours exhibited a short-lasting increase of DNA-synthesis. The maxima of the induced changes in cell cycle phase distribution and DNA-synthesis lasted for only 24-48 hours, which may be of importance for scheduling combinations of drugs. Though both drugs induced profound changes in tumour volume growth and cell kinetics, there was no change in the histopathological picture of the treated tumours. Routine histopathological examination is thus not likely to be of value in order to evaluate the effect of chemotherapy.

Introduction

The low rate of local control and cure in advanced squamous cell carcinoma of the head and neck constitutes a therapeutic problem. Recently, Mead & Jacobs (1982) have discussed this problem and stressed the potential role of chemotherapy in the multimodality treatment.

Chemotherapeutic agents interfere in the metabolism of the malignant cell at different levels. The effects may result in alterations in cell kinetic parameters such as rate of cell proliferation and cell cycle phase distribution. Analyses of these cytokinetic perturbations in experimental models, either *in vivo* or *in vitro*, may give a better basis for monitoring cancer chemotherapy, whether given as single drug or combination of drugs.

Human tumours transplanted to nude mice as a model for chemotherapeutic treatment were originally described by Povlsen et al (1973). Recently this test system has been evaluated for head and neck carcinomas (Wennerberg et al, 1983; Wennerberg, 1983) with respect to differences and similarities between heterotransplants and tumours *in situ*. Though differences in 3HTdR-incorporation into DNA, volume doubling times and clonal composition were found, the cell cycle times for heterotransplants were close to their human counterparts (Frindel et al, 1968; Bresciani et al, 1974; Tannock, 1978). This makes the nude mouse model suitable for studies on some important aspects of chemotherapeutically induced changes as e.g. the course of perturbations in cell cycle phase distribution.

The purpose of the present study was to define alterations in different cell kinetic parameters in a squamous cell carcinoma of the head and neck heterotransplanted to nude mice and treated with chemotherapeutic agents.

Thus, a poorly differentiated squamous cell carcinoma of the head and neck, serially transplanted in nude mice, was treated with either mitomycin C or cisplatin, both alkylating agents used in head and neck cancer chemotherapy (Andersson et al, 1979; Wittes et al, 1979; Glick et al, 1980; Vogl et al, 1982). Dose-response relationship, cell cycle phase distribution and rate of DNA synthesis were studied. These parameters were considered in relation to changes in histopathology and in tumour volume.

Materials and Methods

TUMOUR: The tumour studied was a heterotransplanted, poorly differentiated squamous cell carcinoma of the nasal cavity. It had been transplanted through 15 passages of nude mice before the study. The tumour had histologically a stable morphology and a stable doubling time of 76.4 $\pm$ 2.9 hours (SEM) during serial transplantation. The tumour was aneuploid and remained stable also in this respect.

In some cases s.c. inoculation resulted in the development of two tumours separated from each other. Analysis of growth pattern did not reveal any host induced uniformity in growth. This is in accordance with the findings of Warenius et al (1980) and Spang-Thomsen et al (1980). Thus, in some experiments mice with two tumours were used.

At drug administration the tumours had a median volume of 137 mm^3 (range: 56-575 mm^3). This tumour size was usually reached within three weeks after transplantation.

MICE: For the heterotransplantations five to eight week-old male and female BALB/c nude (nu/nu) mice were used. The colony was kept under sterile but not specific pathogen-free conditions (Wennerberg, 1983).

DRUGS: Mitomycin C (MMC) and cisplatin (cis-diamminedichloroplatinum II; CDDP), obtained as commercially available preparations (Bristol Laboratories) and physiological saline were used for i.p. injections on tumour-bearing animals. The drugs were diluted with sterile water to proper concentrations immediately before administration in volumes of 0.01 - 0.02 ml/g b.wt. The correspondning doses for MMC and CDDP were 1 - 6 and 2.5 - 12.5 mg/kg b.wt. respectively.

TUMOUR VOLUME MEASUREMENTS: Two perpendicular diameters of the tumour were measured with graduated calipers. The tumour volume was then calculated according to the formula for a rotating elipsoid:

$$\text{Volume} = \frac{\text{length} \times \text{width}^2}{2}$$

Tumour volume calculated according to the formula correlates well with measured tumour volume (Osieka et al, 1977; Fodstad et al, 1980).

Tumour diameters were measured at regular intervals. The chemotherapeutic induced growth retardation was quantified as Relative Tumour Size (RTS), i.e. the tumour volume one week after drug administration in relation to the tumour volume at the time of drug injection (Lesser et al, 1980). The formula is:

$$RTS = \frac{\text{Volume } (t = 1 \text{ week})}{\text{Volume } (t = 0)}$$

RTS-values were calculated for the different dose levels and transformed employing the square root of RTS in order to achieve normality (Lesser et al, 1980). Dose-response curves then could be constructed.

CELL KINETIC ANALYSIS: The doses 4 mg MMC/kg b.wt. and 10 mg CDDP/kg b.wt. were chosen for the subsequent cell kinetic studies. Tumour bearing animals were injected with either MMC or CDDP as single doses. Tumours were taken to flow cytometric (FCM) analysis, measurement of DNA synthesis and light microscopical histopathological examination prior to and at different times after MMC (3-168 hr) or CDDP (3-504 hr) administration. The CDDP treated tumours were observed for a period of three weeks (= 504 hrs) in order to reach a conceivable phase of resumed tumour growth following a period of growth arrest. The corresponding period of time for MMC was one week (168 hrs).

The tumour samples for FCM analysis were kept frozen at -70°C in dimethylsulfoxide/citrate buffer. For the preparation of nuclei for flow cytometric DNA analysis the tumour pieces were thawed and immediately brought into a single cell suspension. They were minced with sharp scissors and mashed through a nylon net (pore size 140 µ). Tris-buffer (pH 7.5) was continuously poured over the net during the procedure. The tumour-cell suspensions were centrifuged for 15 minutes at 250 g at 20°C. The cells were fixed in absolute alcohol at -20°C. Fixed cells were exposed to 0.1% RNAse (Sigma R5125, dissolved in 5.5% of 1 N HCl) for 5 minutes at 20°C. The cells were centrifuged and treated with 0.5% pepsin (Merck) for 10 minutes at 37°C, after which the cells were cooled, centrifuged and resuspended in Tris-buffer to a concentration of about 10^6 cells per ml. One ml of the suspension was stained with 0.005% propidium iodide (Calbiochem) (Krishan, 1975) and 0.6% Nonidet (Merck) dissolved in Tris-buffer. Before flow cytometric analysis in a Cytofluorograf System 50-H (Ortho Instruments, USA) the stained cells were filtered through a 50 µ nylon net.

An argon-laser, excitation 488 nm by 450 mW, was used for measurements. Doublets of cells were excluded by electronical threshold settings. Ten to 100 thousand cells were counted in each individual sample.

The method of evaluation of the DNA histograms is described in Fig. 1. The compound DNA histogram can be regarded as made up by individual DNA-distribution curves for the G0+G1, S and G2+M cell populations. The G0+G1 and G2+M populations are estimated from the outer areas of each peak, and the S population is calculated on the base of those estimations and the total amount of cells in the population as shown in Fig. 1. This is likely to result in a slight, systematic overestimation of the G0+G1 and G2+M populations and an underestimation of the S cell population since the areas for G0+G1 and G2+M determination is affected by the presence of S phase cells (Freid, 1976).

The DNA content of mouse lymphocytes was reported to be 82% of the DNA content of human lymphocytes (Handbook of Biochemistry and Molecular Biology, 1976). Thus it is possible to separate normal mouse cells from human diploid cells. The localization of the peak representing the mouse stromal cells of the heterotransplanted tumours is exemplified in Fig. 1.

DNA synthesis was measured as incorporation of tritiated thymidine into tumour DNA applying the technique described by Håkansson & Tropé (1973). The solid tumour was brought into a cell suspension and was incubated for one hour with tritiated thymidine. Nucleosides and nucleotides were extracted with trichloroacetic acid. Counting was done for 5 minutes in a Beckman Liquid Scintillator 7000. The amount of incorporated 3HTdR per unit DNA in the sample was expressed as a logarithmic function. The formula is:

$$\underline{a} = 100 \times {}^{10}\log \frac{CPM \cdot \underline{k}}{DNA}$$

The CPM is the number of counts per minute registered. $\underline{k}$ is a factor needed to correct for inter alia counting efficiency. DNA is the total amount of DNA in the sample determined with the method of Bonting & Jones (1957) and expressed in arbitrary units.

The thymidine uptake in a cell sample determined by the above expression gives near normally distributed variates.

HISTOPATHOLOGIC EXAMINATION: Tissue specimens from the tumours were fixed in 10% neutral formol, dehydrated and embedded in paraffin. Sections, cut at a thickness of 6 µ, were stained in hematoxylin-eosin. Coded slides were histopathologically evaluated in comparison with slides from the primary tumour of the patient.

STATISTICAL ANALYSIS: Analysis of dose-response curves, variations in cell cycle phase distribution and variations in DNA synthesis were done with one way analysis of variance (ANOVA). The number of observations in each experiment are given in LEGENDS.

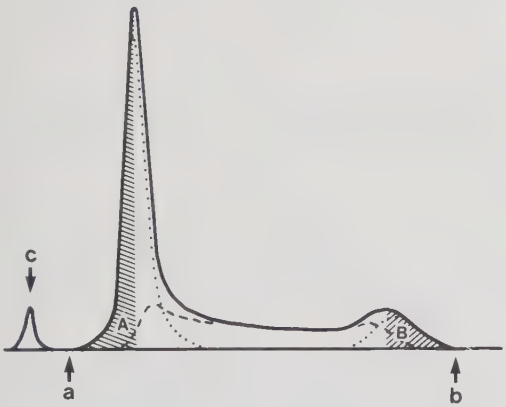

Fig. 1. Schematic drawing of a DNA-histogram of a heterotransplanted tumour. It can be regarded as composed of the DNA-histograms of the G0+G1 (left dotted peak), S (broken line) and G2+M (right dotted peak) cell populations. The fraction of G0+G1 cells is calculated according to the formula N(G0+G1)=2xA, where A is the area at the left half of the G0+G1 peak (shaded). Similarly is the fraction of G2+M cells calculated as N(G2+M)=2xB, where B is the area of the right half of the G2+M peak (shaded). The fraction of S cells is calculated from the total area under the graph between a and b according to the formula N(S)= N(tot) - (N(G0+G1) + N(G2+M)). The single peak marked c represents mouse stromal cells.

Results

FLOW CYTOMETRIC (FCM) STUDIES: Analysis of untreated tumours displayed a steady cell cycle distribution pattern during the observation period. The studied tumour is aneuploid and the DNA content of G0 or G1 cells of the tumour is 1.6 times (DNA-index = 1.6) the amount of DNA in normal human lymphocytes.

In the tumour it was possible to separate the peak of the mouse stromal cells from the peak of human lymphocytes. The DNA content of mouse stromal cells was estimated to 86.9% ± 1.6 (SEM) of the DNA content of human lymphocytes.

3HTdR INCORPORATION STUDIES: Control tumours displayed a steady rate of 3HTdR incorporation into DNA, expressed as a -values during the observation period. The variation in between the tumours were rather pronounced.

EFFECTS OF MMC ADMINISTRATION: The treatment of tumour-bearing animals with MMC (Fig. 2) resulted in a highly significant ($p=0.0004$) dose-dependent retardation of tumour growth, estimated by changes in the square root of relative tumour

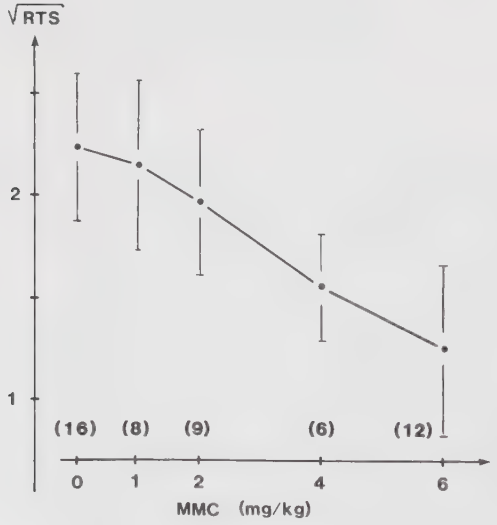

Fig. 2. Dose-response curve for MMC treated tumours. The drug induced tumour effect is quantified as change in $\sqrt{RTS}$, one week after drug administration. (Points = mean, bars = S.D.) Numbers in parenthesis indicate number of tumours examined.

size - $\sqrt{RTS}$ -, one week after drug administration. MMC in the dose 4 mg/kg b.wt. or higher gave a significant inhibition of tumour growth. The dose 4 mg MMC/kg b.wt. was estimated to correspond to LD 10-30.

The drug induced growth disturbances for tumours treated with 4 mg MMC/kg is shown in the growth curve (Fig. 3). MMC caused a retardation of tumour growth, while the controls exhibited exponential growth in the studied volume interval.

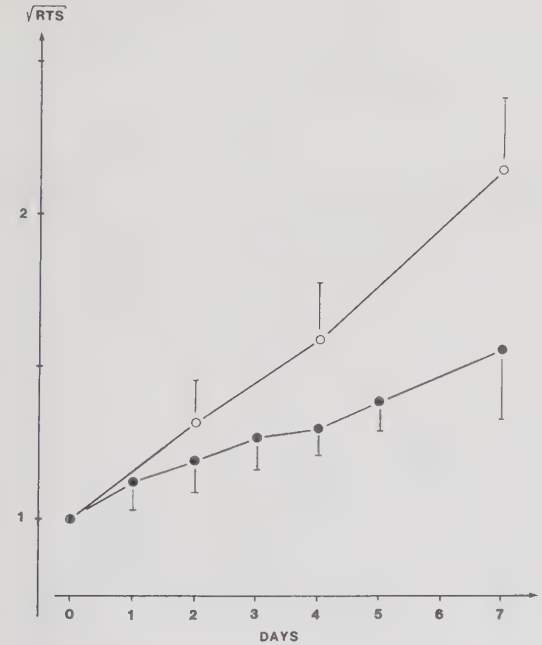

Fig. 3. Growth curves for control and MMC (4.0 mg/kg b.wt.) treated tumours. (Circles = mean of 8 control tumours. Points = mean of 6 treated tumours. Bars = S.D.)

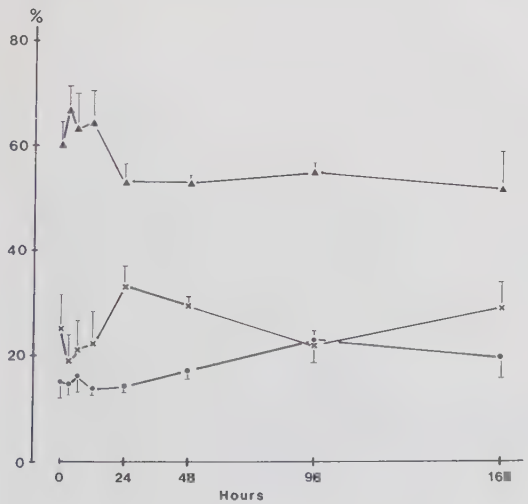

Fig. 4. Cell cycle phase distribution for MMC (4.0 mg/kg b.wt.) treated tumours (▲ (G0+G1), x (S) and ● (G2+M) = mean of 6-12 determinations. Bars = S.D.)

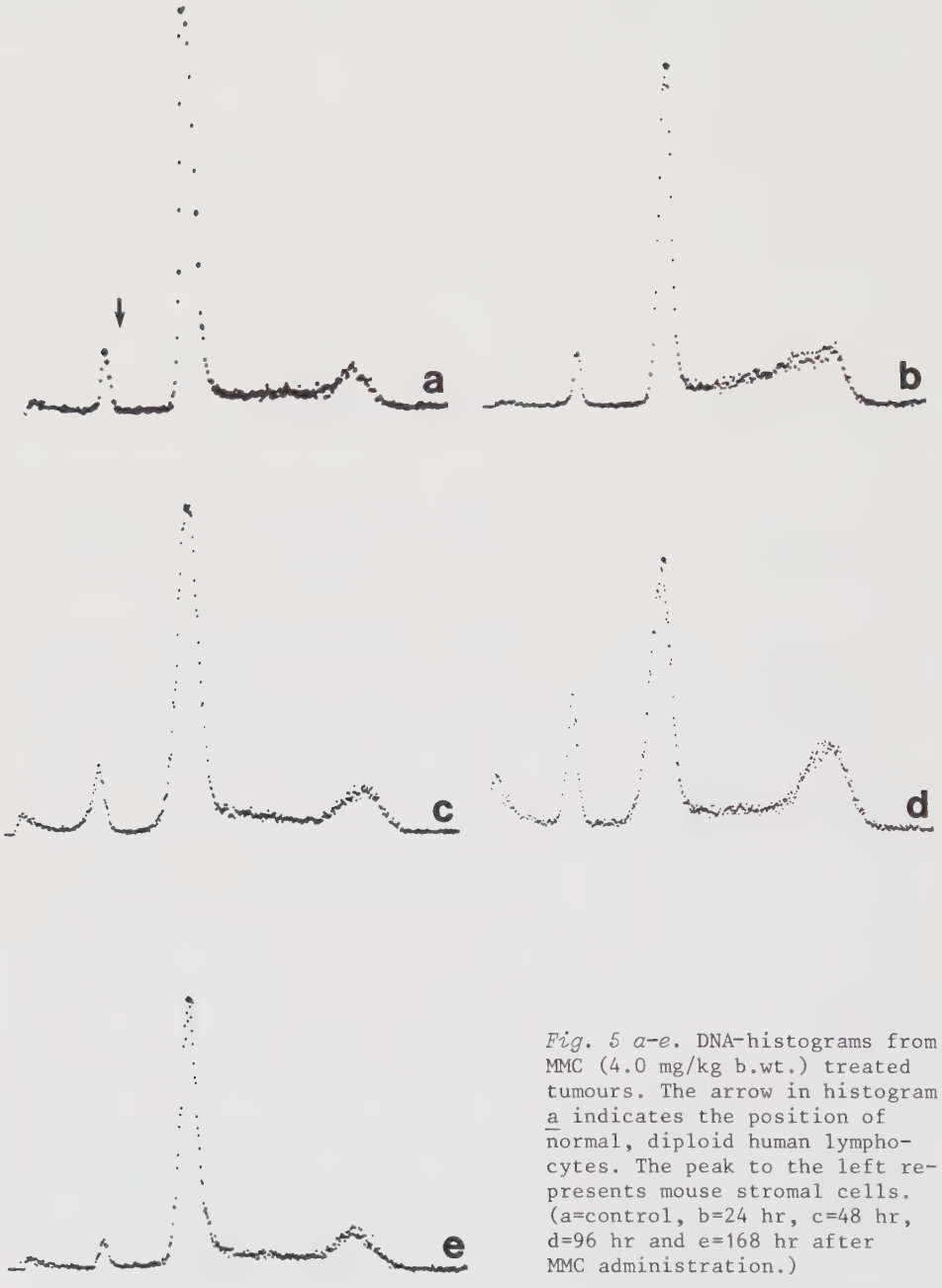

Fig. 5 a-e. DNA-histograms from MMC (4.0 mg/kg b.wt.) treated tumours. The arrow in histogram a indicates the position of normal, diploid human lymphocytes. The peak to the left represents mouse stromal cells. (a=control, b=24 hr, c=48 hr, d=96 hr and e=168 hr after MMC administration.)

FCM analysis showed that treatment with MMC (4 mg/kg b.wt.), after a slight depression of the fraction of S phase cells during 12 hours, resulted in an accumulation of cells in S phase (Fig. 4). Simultaneously there was a small, short-lasting increase of the fraction of cells in G0+G1 phase, followed by a reduction. The changes were most pronounced 24 hours after drug administration. During the rest of the observation period the fraction of cells in G0+G1 remained depressed. After 24 hours the fraction of cells in the S phase compartment declined concomitant with a transient increase of the G2+M compartment, with a maximum after 96 hours. The drug induced cell cycle perturbations are statistically significant ($p<0.05$). The DNA histograms (Fig. 5 a-e) also exhibit a transient accumulation of cells in late S and G2+M phases. The perturbations are most pronounced 24 - 96 hours after MMC administration.

MMC also caused disturbances of the DNA synthesis. A slight depression of 3HTdR incorporation into DNA (Fig. 6), was seen three hours after MMC administration. This reduction was followed by an enhanced rate of 3HTdR incorporation into DNA, with a maximum 12 hours after MMC injection. Twenty-four hours after injection the rate of 3HTdR uptake was normalized and remained so for the rest of the observation period.

EFFECTS OF CDDP ADMINISTRATION: Treatment of tumour-bearing animals with CDDP (Fig. 7) resulted in a highly significant ($p=0.0003$) dose-dependent retardation of tumour growth and CDDP in the dose 7.5 mg/kg b.wt. or higher gave a significant inhibition of tumour growth. The dose 7.5 mg CDDP/kg b.wt. was estimated to correspond to LD 10-20.

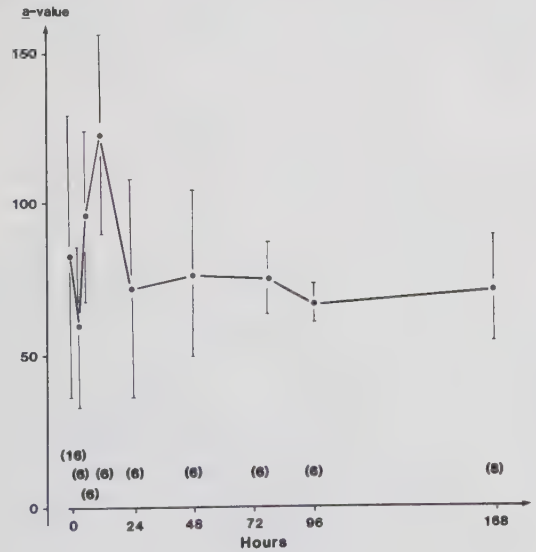

Fig. 6. 3HTdR incorporation in tumour DNA of a MMC (4.0 mg/kg b.wt.) treated tumour expressed as $\bar{a}$-values. (Points = mean, bars = S.D.) Numbers in parenthesis indicate number of tumours examined.

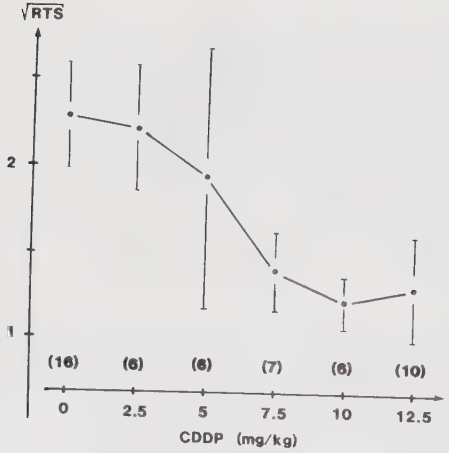

Fig. 7. Dose-response curve for CDDP treated tumours. The drug induced tumour effect is quantified as change in $\sqrt{RTS}$, one week after drug administration. (Points = mean, bars = S.D.) Numbers in parenthesis indicate number of tumours examined.

Fig. 8. Growth curves for control and CDDP (10 mg/kg b.wt.) treated tumours. (Circles = mean of 8 control tumours. Points = mean of 6 treated tumours. Bars = S.D.)

The drug induced growth disturbances for tumours treated with 10.0 mg CDDP/kg is shown in the growth curve (Fig. 8). CDDP results in arrested growth for 12-14 days, after which time volume increment is resumed, though slower than before.

Treatment with CDDP (10 mg/kg b.wt.) resulted in more pronounced cell cycle perturbations (Fig. 9) than MMC administration (Fig. 4). An accumulation of cells in early S phase (Figs. 9 and 10 b), parallel with a decrease of the G0+G1 compartment was registered 24 hours after CDDP administration. The nadir of the G0+G1 depression was reached after 48 hours. Then followed a restitution towards its pretreatment value. An accumulation of cells in S and G2+M phase (Figs. 9 and 10 c) was seen 24 to 48 hours after CDDP administration. The fraction of cells in the G2+M compartment remained elevated for another 48 hours. Forty-eight to 96 hours after CDDP administration the cell cycle phase distribution began to return to normal again. At this time tumour growth was arrested and remained so until 12 - 14 days after CDDP injection. The changes in cell cycle distribution are highly significant ($p<0.0001$).

The sequence of changes is reflected in the DNA histograms (Fig. 10 a-e). Twelve hours after drug injection there is no apparent change in the shape of the DNA histogram. After 24 hours there is an accumulation of cells in early S phase, and after 48 hours the cells have accumulated in late S phase and G2+M phase. Ninety-six hours after drug administration there is still an accumulation of cells in late S and G2+M phases.

CDDP administration also caused disturbances of the DNA synthesis (Fig. 11). A rapid depression of 3HTdR incorporation was seen during the first twelve hours. It was then resumed and normal values were registered 24 to 48 hours after CDDP injection. A second, lower and more slowly developing depression of 3HTdR-incorporation into DNA reached its minimum 168

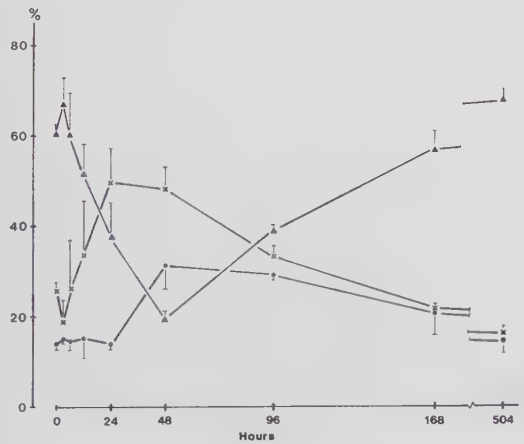

Fig. 9. Cell cycle phase distribution for CDDP (10 mg/kg b.wt.) treated tumours. (▲ (G0+G1), x (S) and ● (G2+M) = mean of 6-12 determinations. Bars = S.D.) Note that the X-axis is broken between 168 and 504 hours.

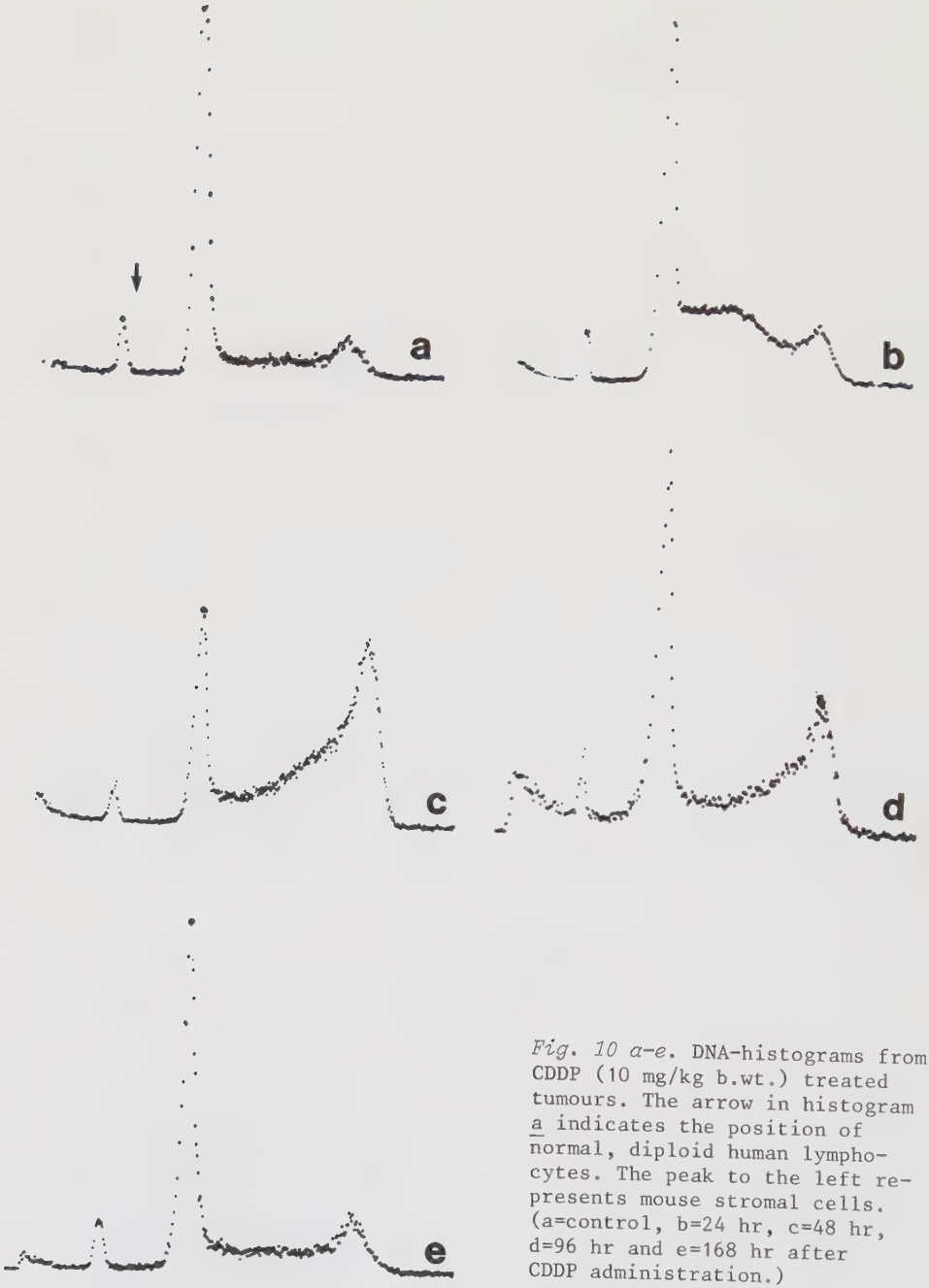

Fig. 10 a-e. DNA-histograms from CDDP (10 mg/kg b.wt.) treated tumours. The arrow in histogram a indicates the position of normal, diploid human lymphocytes. The peak to the left represents mouse stromal cells. (a=control, b=24 hr, c=48 hr, d=96 hr and e=168 hr after CDDP administration.)

hours (= 7 days) after drug administration. It was parallel to the growth arrest of the tumours. The normal level of 3HTdR incorporation was then restituted and even a slight elevation above the pretreatment value (Fig. 11) was documented 21 days after CDDP injection.

LIGHT MICROSCOPIC FINDINGS: No apparent morphologic differences were found between the primary tumour and the transplanted tumours, irrespective of type of treatment and time after treatment. Thus, the tumours were still poorly differentiated squamous cell carcinomas. Mitoses, often of abnormal types, were seen rather frequently. No foci of keratinization were found. Further, necroses, bleedings or infiltrates of inflammatory cells were never found. The amount of stromal connective tissue was always small.

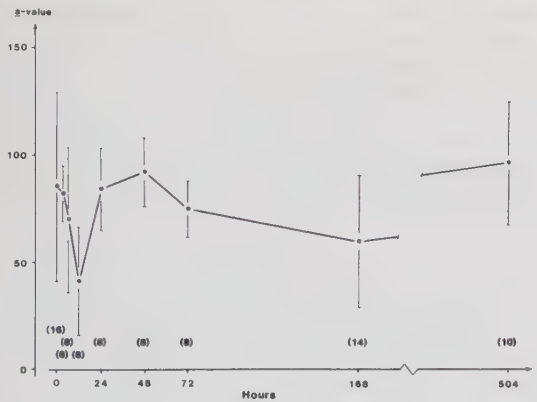

Fig. 11. 3HTdR incorporation in tumour DNA of a CDDP (10 mg/kg b.wt.) treated tumour expressed as a-values. (Points = mean, bars = S.D.) Numbers in parenthesis indicate number of tumours examined. Note that the X-axis is broken between 168 and 504 hr.

Discussion

Cell cycle phase distribution pattern and 3HTdR-incorporation into DNA in malignant tumours are of importance for scheduling of chemotherapeutic agents (Skipper et al, 1978; Tannock, 1978). These parameters may be studied in vivo on tumours in man (Friedman et al 1973, Laerum & Farsund, 1981) or after heterotransplantation to nude mice. However, earlier attempts to clinically monitor cancer chemotherapy by flow cytometric analysis of cell cycle phase distribution have not been successful, the main reason being the complexity of tumour tissue, which besides tumour cells also is composed of diploid inflammatory and other reacting cells, connective tissue and vascular cells, as well as difficulties with interpretation of altered cell cycle distribution caused by drug combinations (Laerum & Farsund, 1981). In contrast to the in situ tumour, heterotransplanted tumours allow controlled analyses of tumour growth pattern before and after chemotherapy (Sordillo et al, 1980). Recently, Wennerberg et al (1983) have studied this model for investigation of tumour cell kinetics of malignant head and neck tumours. However, for heterotransplanted squamous cell carcinomas, either from the head and neck or any other organs, no such scheduling studies seem to have been reported.

Heterotransplanted squamous cell carcinomas of the head and neck differ from the patient tumour with respect to tumour doubling times (Wennerberg, 1983). This has also been found in lung cancer (Mattern et al, 1980) and malignant melanoma (Rofstad et al, 1982). However, in the latter case the difference is not attributable to a shortening of the cell cycle, but rather to a change in cell loss factor (Rofstad et al, 1982). It seems reasonable to admit that this is generally true also in other cases.

In the evaluation of chemotherapeutic induced effects on heterotransplanted tumours, dose-response relationships must first be defined. The doses used in treatment of humans cannot be applied to nude mice since they usually tolerate higher doses than conventional mice and man (Freudenthal et al, 1976; Litterst et al, 1978). In the present study significant tumour growth retardation and tolerable toxicity were achieved at a dose of 4 mg MMC/kg b.wt. and 10 mg CDDP/kg b.wt. However it must be stressed, that chemotherapeutically induced changes of tumour volume may depend on several different events at the cellular level, i.e. cell kill, cell renewal, necrosis, oedema stromal changes etc. In contrast, disturbances at the cellular level may not necessarily be reflected in changes in tumour growth.

In the present study, although tumour growth was retarded, no reduction of tumour volume was found, neither were any disturbances of the histopathological picture found. It is notable that the tumours independent of treatment with MMC or CDDP did not show any detectable morphological changes, although there were profound disturbances in volume growth, cell cycle phase distribution and DNA synthesis. Therefore routine histopathological examination may not be a reliable tool in the evaluation of chemotherapeutically induced tumour effects.

The cytokinetic perturbations were studied after a single dose of the chemotherapeutic agent. Repeated doses were not given as they were likely to give additive effects and changes in growth pattern difficult to analyse.

In the MMC treated tumours the perturbations of the cell cycle phases distribution and 3HTdR-incorporation into DNA are seen when tumour growth is retarded, but not arrested. Also the absence of histopathological changes contradict any significant cell kill.

The sequence of events can be interpreted as a block in the transition of cells between S and G2+M phase, causing accumulation of cells in the S phase during the first 24 hours, after which time the block is released. The return of cells into the cell cycle causes the transient increase in the fraction of G2+M cells. The rise in DNA-synthesis precedes the increase of the fraction of cells in S phase and might represent increased DNA repair after MMC inflicted damage. This change in 3HTdR incorporation could however be an artefarct, since incorporation of tritiated thymidine into DNA might not reflect changes in DNA synthesis. Cytostatic drugs may interact with e.g. salvage pathway enzymes or change the size of the precursor pools. The problem was studied by Håkansson (1975) who investigated the effect of cytostatic drugs on incorporation of labelled precursors into DNA and phosphorylation of thymidine in mouse thymocytes and chick embryo cells. All tested drugs caused a marked depression of incorporation of labelled precursors, but had no influence on the phosphorylation of thymidine. Since stimulation of the synthesis of a precursor pool is a very unlikely action of a cytostatic drug, he concluded that changes in a -values were likely to actually reflect changes in synthesis of nucleic acids.

However, if the changes in DNA-synthesis are considered with respect to the fluctuations in S phase fraction, this synthesis remains depressed, compared to controls, after the inital rise. This indicates a persistent damage to the cell and might explain the exponential, retarded tumour growth.

The described MMC induced changes in cell cycle phase distribution and DNA-synthesis is in agreement with the findings of Barlogie & Drewinko (1980), who treated an *in vitro* cultivated human colon adenocarcinoma cell line (LoVo) with MMC.

The compound changes in cell cycle phase distribution and DNA-synthesis induced by CDDP could be explained by a block of the transition of cells from G1 to the S phase. This should cause the depression of DNA-synthesis during the first 12 hours. The cells are then released and continue through the S phase. This transient G1/S phase block is combined with a more long-lasting block or delay of the transition of cells through G2+M phase. This delay may possibly be explained by a damage to the DNA in the S phase.

If the DNA synthesis is regarded with respect to the concomitant changes in S phase fraction, it does not return to normal after the initial depression but remains lowered and only slowly returns towards normal. The resumed but retarded growth after the phase of growth arrest might reflect this.

The results are in agreement with the findings of Bergerat et al (1979) who studied an in vitro cultivated colon adenocarcinoma cell line (LoVo). They found delayed or blocked cycle traverse in S and G2 phases. After prolonged treatment with high concentrations of CDDP, an additional block in G1 or at the G1 - S boundary was established.

When the tumour growth is resumed the DNA synthesis also tends to return towards normal values. The normalisation of the cell cycle phase distribution could be explained by cell loss preferentially in late S and G2+M phases. However, not even here are there any histopathological changes that indicate major cell kill. Thus a possible explanation is a slow, continuous release of cells from the G2+M compartment into a retarded cell cycle.

The MMC and CDDP induced accumulation of cells in S and G2+M phases theoretically results in an increase of the average cell size, since S and G2+M phase cells are larger and contain more cytoplasm than G0+G1 cells. This must be taken into consideration in the interpretation of the growth curves (Figs. 3 and 8). The chemotherapeutically induced retardation of cellular growth is thus likely to be greater than the measured retardation of volume increment. Thus it is possible to have a reduction in cell number although the tumour volume is still slowly increasing.

Both MMC and CDDP have alkylating properties, and though the changes in cell cycle phase distribution caused by CDDP are more pronounced than for MMC, there are similarities in the pattern of changes. However, the disturbances in DNA-synthesis are quite different with the two drugs, indicating differences in their mode of action.

In the present study it is of interest to note that the maxima of the induced changes in cell cycle phase distribution and DNA-synthesis only lasted for 24 - 48 hours. This emphasizes the importance of scheduling in the combined chemotherapeutic treatment in order to achieve maximal effect and minimal

toxicity. Since it is reasonable to believe that cell cycle times in heterotransplants and human tumours in situ are close to each other (Rofstad et al, 1982; Wennerberg, 1983), the nude mouse model may give indications about optimal treatment intervals in chemotherapeutic regimens.

Acknowledgements

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BLEOMYCIN INDUCED CHANGES IN GROWTH AND CELL KINETICS OF A HETEROTRANSPLANTED SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Abstract

Bleomycin (BLM) is used against squamous cell carcinomas, often in combination with other drugs in phase-specific schedules, since it has been considered a synchronizing agent. However, the reported effects on the cell cycle are contradictory. In the present study a poorly differentiated squamous cell carcinoma of the head and neck, heterotransplanted to nude mice was used for evaluation of BLM induced suppression of growth, and, measured with flow cytometric technique, cell cycle traverse and cell cycle phase distribution. BLM was administered once daily for four days. The dose-response curve exhibited a plateau-phase beginning at a total dose of 200 mg BLM/kg. A single dose of 75 mg BLM/kg caused a transient accumulation of cells in G0+G1 phase and was followed by a synchronized passage of cells through the cell cycle. However, the induced perturbations were small. Cell kinetic analysis of tumours treated with repeated doses of BLM did not result in increased cell cycle perturbations. Thus the present study could not confirm any major cell synchronizing effect of BLM on poorly differentiated heterotransplanted human squamous cell carcinoma.

Introduction

Bleomycin (BLM), a glycopeptide antibiotic originally isolated by Umezawa et al (1966) from Streptomyces verticillus, has an established efficiency against squamous cell carcinomas of the head and neck and plays a major role in combination chemotherapy (Glick et al, 1980).

BLM is used in combination with other drugs in phase-specific schedules, since it has been considered a synchronizing agent with the ability to block cells reversibly in G2 phase as shown in Chinese hamster ovary cells in vitro (Barranco & Humphrey, 1971) and malignant melanoma in man (Barranco et al, 1973). However, in neither a study of two previously operated and irradiated human squamous cell carcinomas (SCC) of the head and neck (Ganzer, 1978) nor in mouse epidermal cells (Thorud & Clausen, 1980) was it possible to confirm this BLM induced synchrony of the cell cycle traverse. Although BLM has long been used in the treatment of squamous cell carcinomas of the head and neck, there does not seem to be any flow cytometric in vivo studies on the effect of the agent on the cell cycle traverse in these tumours.

In the design of pretreatment combination chemotherapy not only clinical experimental data and clinical experience but also results from experimental tumour systems should be taken into consideration (Ervin et al, 1981). Thus for this study a poorly differentiated SCC of the head and neck transplanted into nude mice was used, since previous results have shown that the nude mouse system can be suitable for studies of chemotherapeutically induced changes in cell kinetics of SCC (Wennerberg, 1983; Wennerberg et al, 1983).

The aim of the present study was to analyse, after determination of dose-response relationship, BLM induced changes in the cell cycle traverse measured as perturbations in growth and cell cycle phase distribution in SCC of the head and neck.

Materials and Methods

TUMOUR: The tumour studied was a heterotransplanted, poorly differentiated squamous cell carcinoma of the nasal cavity, passed through 30 generations of nude mice before the study. The tumour was aneuploid and had not changed in histology or in tumour volume doubling time (DT) (79.8 $\pm$ 4.9 (SEM) hours) during serial passages.

At retransplantation of 2x2x2 mm tumour pieces s.c. inoculation in some cases resulted in development of two tumours separated from each other. Since we in accordance with the findings of Warenius et al (1980) and Spang-Thomsen et al (1980) have not found any important degree of host induced uniformity in growth, mice with two tumours in some cases were used in the experiments.

DRUGS: Bleomycin was obtained as commercially available preparation (Lundbeck). The doses administered were diluted with sterile water to suitable concentrations immediately before i.p. administration in volumes of 0.01-0.02 ml/g b.wt.

MICE: Five to eight week-old male and female nude mice BALB/c were used. The colony was kept under sterile but not specific pathogen-free conditions (Wennerberg, 1983).

TUMOUR MEASUREMENTS: Tumour volume was calculated from diameter measurements according to the formula. Volume = length x width^2/2, as previolusly described (Wennerberg et al, 1983). Tumours became palpable two to three weeks after inoculation and were measured regularly.

DOSE-RESPONSE RELATIONSHIP: Tumour bearing nude mice in groups of 6 to 8 animals were treated with either BLM or with 0.9% NaCl. One injection was given daily for four days. The total doses given were 100, 200 and 300 mg BLM/kg b.wt. Tumour diameters were measured regularly during the observation period of 14 days. At the start of drug administration the tumours had a mean volume of 213 $\pm$ 89 mm^3.

The chemotherapeutically induced growth retardation was quantified as Relative Tumour Size (RTS), i.e. the tumour volume after drug administration in relation to the tumour volume at the time of the first drug injection. The formula is:

$$RTS = \frac{\text{Volume } (t = \text{day } n)}{\text{Volume } (t = 0)}$$

RTS-values were calculated for each diameter measurement. The data was transformed employing the square root of RTS, in order to achieve normality. For each tumour the data was displayed as the graph of the square root of RTS versus time, and the area under the graph was taken as a measure of tumour growth, as described by Lesser et al (1980).

CELL KINETIC STUDY: Tumour-bearing animals were injected with either BLM or with 0.9% NaCl. One group of animals was given BLM as a single dose of 75 mg/kg b.wt., another group of animals was given 75 mg BLM/kg b.wt. daily during four subsequent days, i.e. a total dose of 300 mg BLM/kg b.wt. Prior to, and at different times after single dose (3-346 hr) or iterated (3-168 hr) drug administration, 6 - 10 tumours were taken to flow cytometric analyses of DNA content of individual cells.

FLOW CYTOMETRIC (FCM) ANALYSIS: While awaiting FCM analysis the tumour pieces were frozen at -70^{o}C in dimethylsulfoxide/ /citrate buffer. The preparation procedure applied is in detail described by Wennerberg et al (1983). Ten thousand to 70.000 propidium-iodide stained tumour cell nuclei were measured in each individual sample in a Cytofluorograf System 50-H (Ortho Instruments, USA).

The method of evaluation of the DNA histograms is previously described (Wennerberg et al, 1983).

STATISTICAL EVALUATION: The analysis of the dose-response curve and cell cycle phase distribution curves was done with analysis of variance (ANOVA).

Results

DOSE-RESPONSE RELATIONSHIP: Untreated tumours exhibited an exponential growth (Fig. 1) in volume intervals ranging between 100 and 6000 mm^3. There was an increased depression of tumour growth with increasing doses up to 200 mg BLM/kg. However, a further increase of dose to 300 mg BLM/kg did not give any additional effect on tumour growth. The dose 200 mg BLM/kg b.wt., approximately, corresponded to LD 10-20.

The dose response curve was concave and exhibited a plateau--phase (Fig. 2). When growth retardation was estimated as changes in area under the growth curve, the dose-dependent depression of tumour growth by BLM was highly significant ($p<0.00005$). However, when the individual dose levels were compared there were no significant differences in growth retardation between the doses 100 mg and 200 mg BLM/kg, and the between doses 200 mg and 300 mg BLM/kg respectively.

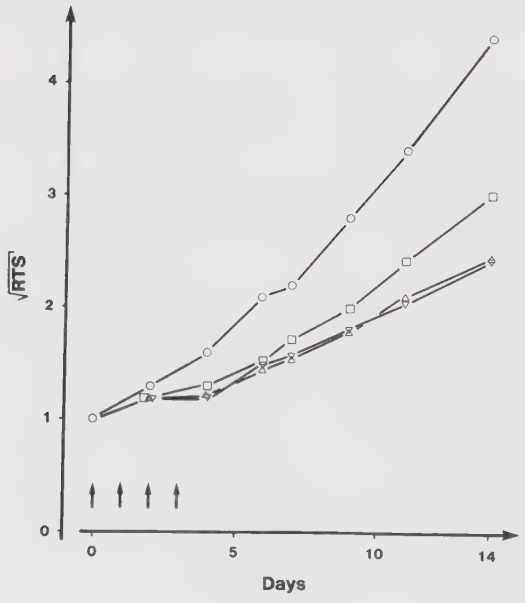

Fig. 1. Growth curves for heterotransplanted tumours treated with total doses of 100 (□), 200 (△) and 300 (▽) mg BLM/kg b.wt. or with 0.9% NaCl (○) given i.p. once daily for four days. (Points = mean of 6-10 tumours.)

CELL-KINETIC RESULTS: A single dose of 75 mg BLM/kg b.wt. resulted in an initial, short-lasting accumulation of cells in G0+G1 phase 3-9 hours after drug administration (Fig. 3). It was followed by a loss of cells from the G0+G1 fraction, a transient accumulation of cells in S phase 48 hours after BLM administration. Forty-eight hours later there was a rise in the fraction of cells in G2+M phase. These changes in cell--cycle phase distribution reached their maxima within 96 hours after drug administration. After this was observed a successive depletion of G2+M cells to a level below the pretreatment value, concomitant with an accumulation of cells in G0+G1 phase at the end of the observation period. The changes in cell cycle phase distribution were statistically significant ($p<0.01$).

BLM in the dose 75 mg/kg/day for four days also induced a tendency to a short-lasting initial accumulation of cells in G0+G1 phase, but otherwise did not show any detectable pattern of cell synchronization or cell cycle perturbation ($p>0.05$).

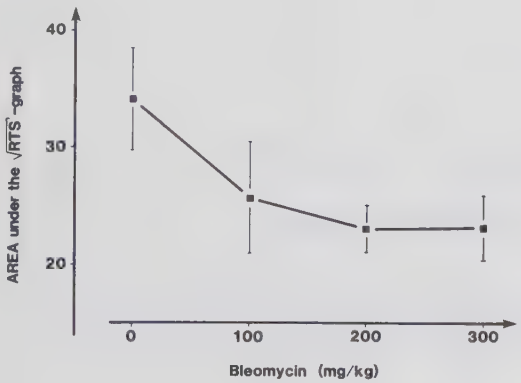

Fig. 2. Dose-response curve for BLM treated tumours. The total doses are given on the X-axis. Tumour growth is quantified as the area under the square root of RTS vs. time graph for two weeks, and is given on the Y-axis. (Points = mean of 6-10 tumour determinations. Bars = S.D.)

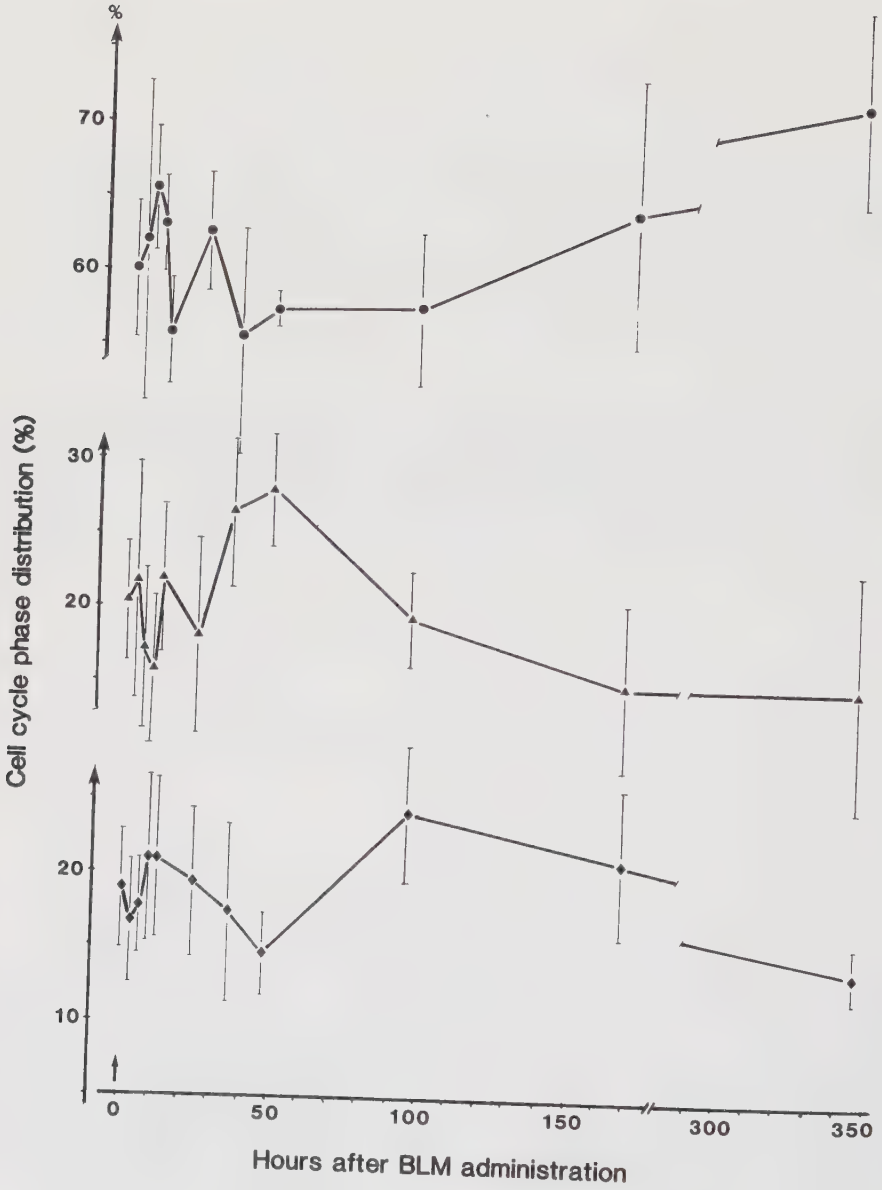

Fig. 3. Cell cycle phase distribution in percent after treatment with 75 mg BLM/kg b.wt. i.p. in single dose. The time scale is divided. ● G0+G1, ▲ S and ◆ G2+M phase. (Points = mean of 6-10 determinations. Bars = S.D.)

Discussion

In the present dose-response study BLM was administered once a day for four days, since previous studies in vitro have indicated that a fractionated scheme was more advantageous than single dose administration (Terasima et al, 1976 a). A four day schedule was chosen, a period covering at least the time of a cell cycle (Tc), which is less than the tumour volume doubling time (DT) of the tumour, in this case 79.8 hours.

Though BLM induced significant retardation of tumour growth, tumour regression was not found (Fig. 1). This is in contrast to the results of Povlsen et al (1973) who treated a well differentiated squamous cell carcinoma of the maxilla heterotransplanted to nude mice, with BLM (6 injections/48 hours/week in 3 weeks) and achieved reduction of tumour volume. The difference in result might be attributed to different administration schedules and/or the difference in degree of differentiation of the treated tumours. BLM has been reported to be more efficient against well differentiated than poorly differentiated SCC (c.f. Glick & Taylor, 1981). The BLM induced decrease of the slopes of the growth curves (Fig. 1), i.e. increase in DT, can be attributed either to an increase in cell cycle time, which has been demonstrated in vitro for mouse L5 cells (Katsumata et al, 1973), or an increase in cell loss in Ehrlich ascites carcinoma (Nagatsu, 1972) and in normal mouse epidermal cells (Thorud & Clausen, 1980).

The concave shape and plateau-phase of the dose response curve which is also found in BLM treated in vitro cultured cells (Terasima et al, 1976 a), may depend on genetically resistent clones (Håkansson & Tropé, 1974) or differences in BLM sensitivity between cycling and non-cycling cells (c.f. Terasima et al, 1976 b). It may also be explained by differences in sensitivity between different cell cycle phases (c.f. Tannock, 1978). Since BLM in man has a half-life (t1/2) between 1.3 and 2.6 hours (Bitter, 1976; Broughton et al, 1977; Oken et al, 1981), and is also likely to be rapidly cleared in nude mice (Freudenthal et al, 1976; Litterst at al, 1978) only the fraction of cells in sensitive phase during drug exposure are killed or damaged. Enhanced drug efficacy may therefore rather be achieved with continuous drug exposure than increase of individual doses (Tannock, 1978). Thus the finding of a plateau-phase pharmacokinetically supports the concept that low dose BLM treatment and continuous infusion may improve the therapeutic index (Krakoff et al, 1977; Sikic et al, 1978).

The flow cytometric data of the present study indicates that a single dose of BLM causes a transient accumulation of cells in G0+G1 followed by a subsequent, synchronized passage of cells through the cell cycle. However the induced perturbations are small (Fig. 3). The cell cycle perturbation may be explained by a reversible arrest of the cells on the G1 - S boundary, followed by a synchronized progression of cells through the cell cycle. Repeated doses of BLM only caused small cell cycle perturbations, which is noteworthy since Barranco et al (1973) after continuous infusion of BLM in malignant melanoma in man noted profound disturbances of the cell kinetics.

However, BLM has been claimed to be a synchronizing agent which at low doses reversibly inhibit cell progression at the S - G2 boundary when studied in vitro in Chinese hamster ovary cells (Barranco & Humphrey, 1971), in vivo in solid Ehrlich carcinoma (Schumann et al, 1975) and malignant melanoma in man (Barranco et al, 1973). It has also been used clinically in the treatment of squamous cell carcinoma (SCC) with this intention (Costanzi et al, 1976; Miyamoto et al, 1978; Biörklund et al, 1982; Tropé et al, 1983). However, other authors in studies of normal mouse epidermal cells (Thorud & Clausen, 1980) and in human SCC of the head and neck (Ganzer, 1978) have not been able to verify such an effect. Though they demonstrated an accumulation of cells in G2 phase, many of the cells accumulated in G2 phase seemed to stop proliferation and die in this phase. This might explain the depletion of the fraction of cells in G2+M phase at the end of the observation period that is seen in this study (Fig. 3). Thorud & Clausen (1980) also found indication of an inhibition of cell cycle progress in either G1 phase or at the G1 - S boundary shortly after BLM administration. A finding which is in accordance with the results of this study. A G1 - S block is also supported by preliminary results from treatment of a poorly differentiated heterotransplanted SCC with continuous BLM infusion for seven days. This resulted in profound cell cycle perturbations with G0+G1 accumulation and S phase depletion during infusion (Wennerberg et al, unpublished data). The divergence in the reported results of BLM on the cell cycle, might be explained by differences in action of BLM between histologically different types of cells.

In conclusion, BLM has been claimed a synchronizing agent with the ability to block cells reversibly in G2 phase. The present study has not confirmed any such block, nor any major cell synchronizing effect on SCC when BLM is administered in single or iterated doses, although there was an overt effect on tumour volume growth. If any, there is rather a G1 - S than a S - G2 block, a finding which is supported by preliminary data from continuous infusion. This emphasizes the importance of dose scheduling and cell kinetic investigations in the treatment of malignant tumours with chemotherapeutic agents. Thus, studies on human tumours heterotransplanted to nude mice can provide valuable information on chemotherapeutic induced cell kinetic changes.

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